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Polynucleotide(s) encoding a QM-type protein in plants - used to transform plants to alter normal development partic. for producing male sterile plants.
Patent Assignee: PIONEER HI-BRED INT INC (PION-N)
Inventor: CIGAN A M; NEILL J D; PIERCE D A; NEILL J
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Abstract (Basic): WO 9421804 A
(A) an isolated polynucleotide molecule is claimed which encodes a

developmental protein in a plant and which is capable of hybridising to a mammalian QM gene.

USE - Recombinant DNA comprising the developmental protein coding sequence can be used to transform plants to alter the normal development (claimed) and to produce male sterile plants (claimed) e.g. for prodn. of hybrid seed. The isolated protein can be used to prepare antibodies for the diagnosis of developmental problems and the analysis of developmental pathways in plants. The protein can also be used to analyse protein interactions during development.

In an example, at least 2 QM polynucleotides and as many as six, were distinguishable by Northern blot analysis of maize prepns. The amino acid sequence of a protein encoded by a polynucleotide was determined. Oligonucleotide primers developed from this sequence were used to amplify the DNA in the open reading frame. The cDNA clone obtd. was 939 nucleotides in length and contained a single open reading frame encoding a polypeptide of 25, 138 daltons.

Dwg.0/25

Title Terms: POLYNUCLEOTIDE; ENCODE; TYPE; PROTEIN; PLANT; TRANSFORM; PLANT ; ALTER; NORMAL; DEVELOP; PRODUCE; MALE; STERILE; PLANT

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(71) Applicant (*for all designated States except US*): PIONEER-HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): NEILL, John, D. [US/US]; 4106 Rose Hedge Drive, Des Moines, IA 50310 (US). PIERCE, Dorothy, A. [US/US]; 9124 Hammontree Avenue, Urbandale, IA 50322 (US). CIGAN, Andrew, M. [US/US]; 875 NW 68th Avenue, Des Moines, IA 50021 (US).

(74) Agents: BENT, Stephen, A. et al.; Foley & Lardner, Suite 500, 3000 K Street, P.O. Box 500, Washington, DC 20007-8696 (US).

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GCCGGCGCCGA CCGTTGAAAG
CTCCGCGAGC TTCTCCACCA GACCAGACGA CGAGCCCGCG NCGTCCGACA
TGTCACGCGC GGGACTCTCT AGGTTTCGTT GTGAGGGGGC GAGCGCGGTG
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ACGCTCATAA TTTAGAATCC GTGTAGACCT TTATCTGTCA AAAAAGGGGA
GTATTAGTCA TATTCCATGG
NcoI

(57) Abstract

A family of genes has been found in plants, said genes encoding a family of developmental proteins that have homologous structures to the mammalian QM genes. A QM promoter and anther specific promoters have been isolated. Recombinant molecules which include the QM genes in plants are useful to transform cells and regenerate plants that, as a result, have altered developmental pathways. Methods of producing male sterile plants use recombinant molecules containing either the QM sense genes or antisense genes with appropriate promoters.

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METHODS AND COMPOSITIONS FOR CONTROLLING PLANT DEVELOPMENT

BACKGROUND OF THE INVENTION

The structure and genetic coding sequences of a family of developmental proteins in plants have homology to mammalian QM proteins and to genes encoding the proteins. Recombinant molecules comprising plant QM coding regions and suitable promoters, are used to produce a transformed plant with altered development. The altered development causes male sterility.

The expression of most, if not all, plant genes can be considered to be related in some way to plant development. Many classes of genes are known to respond to development signals involved in cell differentiation, formation of tissues and organs, or in controlling plant growth. There are several well-characterized examples: genes that are regulated by light (such as rbcS and cab gene families), or by hormones, and genes that are expressed specifically in anthers, roots, seeds or leaves, or in specific cell types in these tissues. See Edwards *et al.*, 1990, and Kuhlmeier *et al.*, 1987, for reviews. Other types of genes are known to regulate the expression of yet other genes, such as the maize regulatory gene "Opaque2," which codes for a transcriptional activator regulating the expression of 22kd zein genes (Schmidt *et al.*, 1992, Ueda *et al.*, 1992), and the C1 and R genes in maize that code for transcriptional activators which regulate the expression of A1 and BZ1 (Klein *et al.*, 1989). A new area of research relates to the identification and isolation of plant genes which, based on their homology to genes from animal and yeast systems, are believed to be involved in the control of basic cell processes such as cell division. See Jacobs, 1992, for a review. An example of such a gene is the homologue of the yeast *cdc2* gene which has been cloned from maize

(Colasanti *et al.*, 1991). In the future, there are certain to be additional genes identified in plants which control other basic cellular or developmental processes.

In mammals, developmental proteins have been implicated in abnormal cell division such as characterizes the malignant state. For example, Wilms' tumor is a pediatric tumor of the kidney which arises in embryonic blastoma cells and occurs in both sporadic and hereditary forms. Three groups have reported the cloning of two distinct genes which are associated with Wilms' tumor. The first, *WT1*, encodes a zinc finger protein belonging to the early growth response (EGR) gene family and maps to the 11p13 locus in humans, which is often deleted in tumorigenic cells (Call *et al.*, 1990, Gessler *et al.*, 1990). The second gene, designated "QM," was cloned by Dowdy *et al.*, 1991, through the use of subtractive hybridization using cDNAs and RNA derived from tumorigenic and non-tumorigenic Wilms' microcell hybrid cells, respectively. This gene was shown to be expressed at the RNA level in virtually all normal tissues examined in the mouse but was lacking in Wilms' tumorigenic cell lines.

The protein encoded by the QM gene is 25 kD in size and is very basic with a pI of approximately 11.0. Dowdy also demonstrated that QM is a member of a family of genes in a number of mammals, particular primates. van den Ouweland *et al.* (1992) cloned the QM gene from a human Xqter chromosome library and showed that this gene was 100% similar to the previously cloned QM gene. The expression of the QM gene has been demonstrated in the mouse (Dowdy *et al.*, 1991). Information and the gene cloned in the chicken, with data from van den Ouweland *et al.*, suggests that this gene is conserved across a large phylogenetic range.

It has been postulated that QM may be involved in maintenance of the non-tumorigenic phenotype (Dowdy *et al.*, 1991). Recent experiments suggest that QM may act as a negative regulator of the transcriptional activator Jun by competing with other proteins (Fos) that bind to Jun leading to speculation that lack of QM protein leads to unregulated cell growth and ultimately tumor formation (Monteclaro *et al.*, 1993). These results did not suggest that a QM gene might exist in plants, for

which there are not phenotypes comparable to those associated with the QM gene in animals.

Discovery of genes which alter plant development can be useful in developing genetic methods to induce male sterility because other methods currently available, such as detasseling, cytoplasmic male sterility and self-incompatibility, have serious shortcomings. Male sterile plants are useful for production of hybrid seed.

Production of hybrid seed for commercial sale is a large industry. Plants grown from hybrid seed benefit from the heterotic effects of crossing two genetically distinct breeding lines. The agronomic performance of this offspring is superior to both parents, typically in vigour, yield and uniformity. The better performance of hybrid seed varieties compared to open-pollinated varieties makes the hybrid seed more attractive for farmers to plant and, hence, commands a premium price in the market.

In order to produce hybrid seed uncontaminated with self-seed, pollination control methods must be implemented to ensure cross-pollination and not self-pollination. Pollination control mechanisms can be mechanical, chemical or genetic.

A mechanical method for hybrid seed production can be used if the plant species in question has spatially separate male and female flowers or separate male and female plants. The corn plant, for example, has pollen producing male flowers in an inflorescence at the apex of the plant and female flowers in the axils of leaves along the stem. Outcrossing is assured by mechanically detasseling the female parent to prevent selfing. Even though detasseling is currently used in hybrid seed production, the process is not only labor-intensive but also costly, since yield loss is incurred.

Most major crop plants of interest, however, have both functional male and female organs within the same flower, so that emasculation is not a simple procedure. It is possible to remove by hand the pollen forming organs before pollen shed, however, this form of hybrid seed production is extremely labor intensive and, hence, expensive. Seed is produced in this manner only if the value and amount of seed recovered warrants the effort.

A second general method of producing hybrid seed is to use chemicals that kill or block viable pollen formation. These chemicals, termed gametocides, are used to impart a transitory male sterility. Commercial production of hybrid seed by use of gametocides is limited by the expense and availability of the chemicals and the reliability and length of action of the applications. These chemicals are not effective for crops with the extended flowering period because new flowers will be produced that will not be affected. Another problem is that repeated application of chemicals is impractical because of costs.

Many commercial hybrid seed-production systems for field crops rely on a genetic method of pollination control. Plants that are used as females either fail to make pollen, fail to shed pollen, or produce pollen that is biochemically unable to effect self-fertilization. Plants that are biochemically unable to self-pollinate are said to be "self-incompatible" (SI). Difficulties associated with the use of a self-incompatibility system include (i) availability and propagation of the self-incompatible female line and (ii) stability of the self-compatibility. In some instances, self-incompatibility can be overcome chemically, or immature buds can be pollinated by hand before the bio-chemical mechanism that blocks pollen is activated. Unfortunately, self-incompatible systems that can be deactivated are often very vulnerable to stressful climatic conditions that break or reduce the effectiveness of the biochemical block to self-pollination.

Of more widespread interest for commercial seed production are systems of pollen control-based genetic mechanisms causing male sterility. These systems are of two general types: (a) nuclear genic male sterility, caused by the failure of pollen formation because of one or more nuclear genes and (b) cytoplasmic-genetic male sterility, commonly called "cytoplasmic male sterility" or CMS, in which pollen formation is blocked or aborted because of a defect in a cytoplasmic organelle, which generally is a mitochondrion.

Nuclear (genic) sterility can be either dominant or recessive. A dominant sterility can only be used for hybrid seed

formation if propagation of the female line is possible, for example, via *in vitro* clonal propagation. A recessive sterility could be used if sterile and fertile plants are easily discriminated. Commercial utility of genic sterility systems is limited however by the expense of clonal propagation and rouging the female rows of self-fertile plants.

Although there are reports of hybridization schemes involving the use of CMS, there are many problems that limit its commercial value. In these systems, a specific mutation in the cytoplasmically located mitochondrion can, when in the proper nuclear background, lead to the failure of mature pollen formation. In some instances, the nuclear background can compensate for the cytoplasmic mutation and normal pollen formation occurs. The nuclear trait that allows pollen formation in plants with CMS mitochondria is called restoration and is the property of specific "restorer genes." Generally, the use of CMS for commercial seed production involves the use of three breeding lines, the male-sterile line (female parent), a maintainer line which is isogenic to the male-sterile line but contains fully functional mitochondria and the male parent line.

The male parent line may carry the specific restorer genes, usually designated a "restorer line," which then imparts fertility to the hybrid seed. For crops, such as vegetable crops for which seed recovery from the hybrid is unimportant, a CMS system could be used without restoration. For crops for which the fruit or seed of the hybrid is the commercial product, the fertility of the hybrid seed must be restored by specific restorer genes in the male parent or the male-sterile hybrid must be pollinated. Pollination of non-restored hybrids can be achieved by including with hybrids a small percentage of male fertile plants to effect pollination. In most species, the CMS trait is inherited maternally (because all cytoplasmic organelles are inherited from the egg cell only), which can restrict the use of the system.

Although CMS systems are reported, they possess limitations that preclude them as a solution to production of male sterile plants. For example, one particular CMS type in corn (T-cytoplasm) confers sensitivity to infection by a

particular fungus. Although still used for a number of crops, CMS systems have a tendency to break down with prolonged use. Generally, male sterility is less than 100%.

A search for methods of altering development in plants, for example, to produce male sterile plants, revealed an exceptionally suitable family of developmental proteins in plants, the QM family. The methods and compositions of the present invention provide a new nuclear basis for manipulating male fertility.

SUMMARY OF THE INVENTION

The present invention relates methods and compositions for altering plant development. The methods use genetic constructs including the QM gene isolated from plants.

It was unexpected to find a QM gene in plants. The QM gene has been described in mammals in relation to tumors, being expressed in normal cells, but not expressed in tumor cells. The gene is likely to be down-regulated in tumors, for example in Wilms' tumor in humans. A gene related to mammalian oncogenesis would not be expected to have a homologue in plants, because comparable developmental abnormalities do not occur. Tumors are known to occur in certain plant species, but these are specifically caused by infection by exogenous agents such as Agrobacterium or other pathogens. Yet, a polynucleotide was isolated from the maize genome that unexpectedly showed homology with the nucleotide sequence of the mammalian QM gene. That polynucleotide from maize is referred to hereon as "QM_m." A QM gene was also cloned in tobacco (QM_T).

The protein encoded by the maize polynucleotide is a developmental protein. Developmental proteins include proteins that are expressed during development in response to a regulatory signal such as a hormone, and proteins that regulate developmental pathways. The QM gene in plants therefore is useful in the context of controlling development, for example, development of pollen. Interference with pollen development produces a male sterile plant. Developmental proteins are recognized by their ability to alter the result of normal development structure or function.

A cDNA prepared from a QM_m polynucleotide consists essentially of 800-950 nucleotides, including an open reading frame (ORF) and flanking regions. The comparable mammalian CDNA generally is less than 800 nucleotides. The single open reading frame in the maize cDNA encodes a polypeptide of approximately 220 amino acids. In other species, an open reading frame in the QM cDNA isolated from humans, encodes a family of QM protein of approximately 214 amino acids. In general, a QM family of genes in plants (QM_p) encodes a protein characterized by a primary sequence of approximately 200-250 amino acids, and has properties described herein.

More specifically, genes of the QM family encode a family of proteins that is characterized by the presence of three conserved regions in the amino acid sequence of the protein members of the family. In a maize QM protein, the first conserved region includes the first 10 amino acids positioned from the amino terminus; the second conserved region includes the amino acid sequence from residues 50 to 60, and forms an amphipathic helix region in the QM protein; the third conserved region is located at residues 98-135. These three conserved regions exhibit a high degree of homology to corresponding regions that are characteristic of their mammalian counterparts. "High degree of homology" is defined here to denote that at least 80% of the amino acids at corresponding positions, as defined in reference to the amino terminus of the sequence, are identical.

The overall homology of a plant QM amino acid sequence, relative to a mammalian counterpart, is generally at least 50%. (The differences between plants and mammals occur in the region from approximately residue 135 (relative to the N-terminal) to the C-terminal end of the protein.

The nucleotide sequence positions from the N-terminus that encode the conserved region in the maize QM gene are located at approximately positions 30-100, positions 210-250 and positions 330-400. Hybridization probes prepared from these regions will hybridize to the comparable mammalian QM sequences under stringent conditions. Oligonucleotide probes prepared from the conserved region are useful to detect new QM genes in plants under low stringency conditions, for example, using 50%

formamide, 5X SSC (0.75 M NaCl), at 37°C. The coding regions of the maize sequence show approximately 64% homology to the human QM sequence overall.

Because of "wobble" in the third position of each codon in the nucleotide sequence, a functionally similar protein can be encoded with as much as 36% overall divergence between the nucleotide coding regions. In the same species, a sequence encoding at least the three conserved regions is expected to encode a functionally equivalent protein. This is not necessarily true of cross-species comparisons, where protein function is interrelated with biochemical pathways characteristic of the species. But both the plant and the mammalian QM proteins have major effects on developmental processes.

At least two QM polynucleotides, and as many as six, are distinguishable by Northern blot analysis of maize preparations. An illustrative embodiment of a polynucleotide encoding a QM protein is shown in FIG. 1 for maize. The amino acid sequence corresponding to FIGURE 1 is shown in FIGURE 2. Oligonucleotide primers developed from this sequence are used to amplify the DNA in the open reading frame. Oligonucleotide primers are used to amplify the DNA in the open reading frame of claim 2, said primers having the nucleotide sequences: Left: 5'-ATGGGCAGAAGGCCTGCTAGATGC / Right: 5'-CAACGGCATCGAGGAAAGCCTTCC. Primers that are useful in detection of the tobacco homologue include GCGAGATCTAAACCATGGGCAGAAGGCC and GCGAAGCGGCCGCTTAAGCAACGGCATCGAGGAAAGCC. PCR oligonucleotides were used to amplify the tobacco QM gene from generic tobacco (sp. xantni), by means of Taq polymerase (Perkin Elmer), at a hybridization temperature of 55°C, and amplified product was digested with BglII and NotI, electrophoresed on 0.8 LMA, subcloned into Bluescript SK+ and then sequenced.

An isolated and purified plant QM maize protein has an estimated molecular weight of approximately 25 kD and a PI of approximately 11.0. A protein deduced from the cDNA sequence will be free of other proteins when prepared synthetically by recombinant methods. Isolated and purified QM proteins and epitopic fragments thereof are useful in preparing antibodies. These antibodies in turn are useful for diagnosis of

developmental problems and the analysis of developmental pathways in plants. The location and level of expression of the QM protein is useful in determining how to alter development. For example, the antibodies developed to QM are useful to determine if and when the protein is turned on in specific cells or tissues of the plant. This information is useful in developing methods for interfering with or enhancing developmental pathways, including those related to pollen development. Such information is useful in developing superior plants, or male sterile plants, for example.

Isolated and purified QM proteins in plants are also useful in analyzing protein-protein interactions. For these purposes, labeled protein probes are developed. A fusion protein including the QM protein is prepared in *E. coli*, for example, isolated, labelled and used in detecting protein interactions during development. See Smith & Johnson, 1988, and Ron & Dressler, 1992.

A recombinant DNA molecule is prepared comprising the QM gene in the sense orientation, *i.e.*, an orientation such that the normal mRNA is transcribed and is used as a template to translate the normal QM gene protein) and a promoter capable of regulating transcription of said DNA in a plant cell. The recombinant DNA molecule can alternatively encode the QM gene in the antisense orientation. This molecule contains the QM gene cloned in the opposite direction such that the minus or non-coding strand is transcribed. No QM gene product is translated, but a RNA transcript complementary to the QM mRNA is produced which is inhibitory to the translation of the plants own QM mRNA, thus decreasing the amount of QM protein produced.

The promoter in the construct can be a cell- or tissue-specific promoter, so that the gene is be expressed in specific cells or tissues. For example, in a method for producing a male sterile plant, an anther specific or tapetal-specific promoter is preferred. The TA39 promoters which are aspects of the present invention, are a suitable promoter. Anther tissue and tapetal cells are examples of a tissue or cell that is crucial for development of pollen. Anther tissue includes support cells and developing microspores, and excludes mature pollen. A QM gene

construct can be effective in altering development whether expressed in a sense or an antisense orientation. If there are genes and processes in anther tissue which are or can be regulated by QM, a sense QM construct could affect development by altering the timing of regulation by QM or affect development by overexpression of the QM protein. Correspondingly, if QM or any genes which can be regulated by QM are essential for normal anther development, expression of an antisense QM construct could affect development by interfering with normal QM expression.

The promoter in the construct may be an inducible promoter, so that expression of the sense or antisense molecule in the construct can be controlled by exposure to the inducer. Exemplary of such an inducer is a plant hormone used to control a hormone-sensitive promoter. Partial sequence of a QM promoter isolated from maize is shown in FIGURE 3.

Altering development is particularly useful to produce a male sterile plant. A method for producing a male sterile plant is to transform a plant cell with a recombinant molecule comprising the sense gene for the QM plant protein, or an antisense molecule directed to the QM gene. An appropriate promoter is selected depending on the strategy for developmental control. For example, a strategy is to overexpress the QM gene selectively in anther tissue by using an anther specific promoter. To produce a male sterile plant, the transformed cell would be regenerated into a plant, pursuant to conventional methodology.

A transgenic plant containing the QM gene construct can be regenerated from a culture transformed with that same construct, so long as plant species involved is susceptible to regeneration. "Culture" in this context comprehends an aggregate of cells, a callus, or derivatives thereof that are suitable for culture.

A plant is regenerated from a transformed cell or culture, or from an explant, by methods disclosed herein that are known to those of skill in the art. Methods vary according to the plant species. Seed is obtained from the regenerated plant or from a cross between the regenerated plant and a suitable plant of the

same species using breeding methods known to those of skill in the art.

Male sterile tobacco plants were produced by regenerating plants from tobacco leaf explants transformed with either a QM_m sense gene, or QM_m antisense construct. A likely explanation is that the exogenous QM_m gene expression disrupted the normal developmental balance of tobacco male fertility.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate presently preferred embodiments of the invention. Together with the foregoing general description and the following, detailed description of the preferred embodiments, the drawings detailed below serve to explain the principles of the invention:

FIGURE 1. Nucleotide sequence and the encoded amino acid sequence of the cDNA clone 10-15 which encodes the maize QM homolog.

FIGURE 2. Amino acid alignment analysis of the maize QM homolog (upper sequence) with the human QM amino acid sequence.

FIGURE 3. A partial nucleotide sequence of a QM promoter from maize.

FIGURE 4. A plasmid map of the plasmid designated pPHI3621, including a QM_m sense gene.

FIGURE 5. A plasmid map of the plasmid designated pPHI3622, including an antisense to QM_m.

FIGURE 6. A plasmid map of the plasmid designated pPHI1285, a selectable bar plasmid for tobacco.

FIGURE 7. A plasmid map of the plasmid designated pPHI4722, including a human antisense gene.

FIGURE 8. A plasmid map of the plasmid designated pPHI4723, including a human sense gene.

FIGURE 9. A plasmid map of the plasmid designated pPHI4719.

FIGURE 10. A plasmid map of the plasmid designated pPHI4720.

FIGURE 11. A plasmid map of the plasmid designated pPHI687.

FIGURE 12. A plasmid map of the plasmid designated pPHI610.

FIGURE 13. A plasmid map of the plasmid designated pPHI460 including a uidA gene.

FIGURE 14. A plasmid map of the plasmid designated pPHI1952 including a uidA gene.

FIGURE 15. A plasmid map of the plasmid designated pPHI2125 including a uidA gene.

FIGURE 16. A plasmid map of the plasmid designated pPHI1527, including a luciferase gene.

FIGURE 17. A plasmid map of the plasmid designated pPHI3620, including a QM_m sense gene.

FIGURE 18. A plasmid map of the plasmid designated pPHI1493, including a GUS gene.

FIGURE 19. A plasmid map of the plasmid designated pPHI4745 (TA39 (14B1)), including a sense gene.

FIGURE 20. A plasmid map of the plasmid designated L62 (TA39 (14B1)) including an antisense to QM_m.

FIGURE 21. A plasmid map of the plasmid designated pPHI4855 (TA39 (8B3)), including a GUS gene.

FIGURE 22. A plasmid map of the plasmid designated L59 (TA39 (8B3)), including a QM_m sense gene.

FIGURE 23. A plasmid map of the plasmid designated L61 (TA39 (8B3)), including an antisense to QM_m.

FIGURE 24. The nucleotide sequence of the TA39 (8B3) promoter.

FIGURE 25. The nucleotide sequence of the TA39 (14B1) promoter.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention relates to methods and compositions for altering development in plants. The invention also is concerned with a plant gene homologue of a constitutively expressed gene in humans that is thought to play a role in maintenance of the non-tumorigenic state, and that has been shown to be absent in Wilms' tumorigenic cells lines. This gene, which

is present as a gene family in humans and rodents, has been demonstrated in a number of diverse mammalian species.

Pursuant to the present invention, a gene has been cloned from maize and tobacco that encodes a protein having a high degree of homology with the human QM protein (approximately 67%). The maize gene encodes a polypeptide of 25 kD of which basic residues comprise 22% of the protein. This gene is expressed in all maize tissues examined by northern blot analysis and is a member of a plant gene family.

The present invention further relates to a method for producing male sterile plants and hybrid seed, to genetic material employed to impart the male sterility trait, and to new products produced by the method, namely, genetically transformed plants carrying the male sterile trait, male sterile plants and hybrid seed produced by pollinating said plants with pollen from male fertile plants.

FIGURE 1 illustrates the nucleotide and derived amino acid sequence of a clone designated 10-15. The cDNA clone was 936 nucleotides in length and contained a single open reading frame encoding a polypeptide of 25,138 daltons. This polypeptide is very basic, having a calculated pI of 11.0, with the basic residues being distributed throughout the protein. In the search for homology with other previously characterized genes, the amino acid sequence encoded by clone 10-15 was used to survey the GenBank database by means of the TFASTA program of Genetic Computer Group (GCG, Devereux *et al.*, 1984). This analysis yielded a score of 716 with the human QM gene. When the amino acid sequence encoded by clone 10-15 was aligned with the amino acid sequence of the human gene (FIGURE 2), several regions of interest are notable. First, is the high degree of conservation of the amino-terminal region, where the first ten amino acid residues are conserved. The second region, again conserved in the two proteins, is between residues 50 and 61 which forms a putative amphipathic helix. There is a third conserved region from residue 98-135.

With reference to the three conserved regions together, the presence of a 59-residue stretch of highly conserved amino acid implicates a conserved function within these regions of the

protein. The carboxy-terminal region is poorly conserved and may not be as important in the function of the protein. Northern blot analysis of RNA isolated from leaf and root tissues from seven day corn seedlings demonstrates that cDNA from clone 10-15 is expressed in both, with roots and leaves showing roughly the same level of expression. In additional northern blots, this gene was found to be expressed in anthers and earshoots.

Southern blot analysis demonstrates that the maize homolog is a member of a small family of approximately 4 to 6 members in maize.

The following examples illustrate methods of practicing the invention. The scope of the invention is not limited to the embodiments illustrated in these representations.

Example 1: Interference with Normal Development of Tobacco Plants by Transformation with the QM_m Gene

Tobacco seeds (cv. xanthi) were germinated under sterile conditions. After approximately 7 to 10 days under light at 28°C, the cotyledons and first leaves were removed aseptically and cut into fourths (approximately 1-2 mm square sections) and placed onto sterile filter paper discs saturated with medium containing 0.25 M sorbitol. The discs were incubated in the dark at 28°C overnight. The next morning the tissue sections were bombarded by means of a biolistics apparatus to transform cells with an equal mixture of the QM_m construct (pPHI3621-sense construct, FIGURE 4 or pPHI3622-antisense construct, FIGURE 5) and a plasmid containing the selectable marker (BAR gene). Total DNA of 0.1 μg was sent by means of five bombardments.

Following bombardment, the tissue was returned to 28°C incubation in the dark. After 48 hours the bombarded tissue was transferred to selection medium (BASTA) and placed under lights at 28°C. After about 2 weeks, small colonies began to appear, and continued to appear for about 1 week. The leaf pieces were transferred to regeneration medium which allowed leaves and plantlets to form. After the formation of plants, the young plantlets were transferred to rooting medium to allow root

formation. After about 1-2 weeks, the plants were taken to the greenhouse for planting.

The sense construct, pPHI3621 (FIGURE 4) did not yield as many colonies as did the control (selectable marker alone). In fact, many colonies formed, but subsequently died. Those that lived grew at a much slower rate than the controls. Most of the surviving calli generated from the colonies did not give rise to plants. Most of those that did produce plants did so from growth of a distinct portion of the calli indicating a revertant sector (loss of the plasmid). The resulting plants were negative for the maize gene by PCR analysis. Observations on the calli indicates they were having trouble forming or organizing a meristem to produce a plant. A plant was found to be positive for the plasmid, yet did produce a plant. However, this plant grew very slowly and did not produce roots by the time it was transferred to the greenhouse. It grew extremely slowly in the greenhouse for some time (approximately 1 month) after which it grew at a normal rate and appeared normal. The plant flowered and set seed in a fashion similar to normal plants, however, the seeds that were produced were abnormal looking, and in germination tests took greater than two weeks to germinate, compared to 4-6 days for normal seed.

The calli derived from the antisense (pPHI3622) (FIGURE 5) bombarded tissues showed completely different growth characteristics. The calli in several instances grew at a much accelerated rate and produced an abundance of vegetative growth. These calli produced plants at a near normal rate. The plantlets moved to regeneration and rooting medium produced roots at a rate faster than controls. The resulting plants appeared normal, flowered and set seed in a normal fashion. The seeds produced germinated normally and the plants appear normal.

These results suggest the QM gene plays a role in development. In transformed tobacco, most likely, its presence prevents or inhibits meristem formation. When expressed, QMs may "fix" a cell at a specific developmental stage. After the gene is turned on, the cell will no longer differentiate. Overexpression of the gene in tobacco calli inhibited the

formation of meristem to generate plants. Overexpression may be lethal at higher concentrations.

The plant cells with constructs including the antisense molecules, were able to grow in some cases at accelerated rates. An interpretation of these results is that the antisense molecule was stopping the action of the tobacco QM gene product, and allowing differentiation to occur more readily and to produce the abundance of foliage seen on the calli. The experiment was repeated 3 times and basically the same observations were made in each experiment. These results are consistent with the observation that QM may function as a repressor molecule. See Monteclaro *et al.*, 1993.

Example 2: Demonstration of Microspore-Specific Gene Expression by *In Situ* Hybridization

This example illustrates a method for showing that an isolated DNA comprises a gene that exhibits microspore-specific expression. In particular, the results here demonstrate that expression of mRNAs related to a particular tobacco cDNA clone is localized to microspores of tobacco anthers. "Microspore specific" as used herein relates to gene expression in any cell or tissue that is crucial for pollen development. Anthers and tapetum are examples of such cells or tissues.

An anther-specific tobacco cDNA clone, designated TA39, was obtained from Dr. Robert B. Goldberg of the Department of Biology, University of California, Los Angeles, California. The clone was then used to recover the TA39 promoter to be used with the QM gene. (See Example 3). This cDNA hybridizes to mRNA from tobacco anthers and not to mRNA from the pistil, petal, leaf or stem (Koltunow *et al.*, 1990). The cDNA is 490 bases long, including a poly A+ tail of 42 bases. This cDNA hybridizes to two transcripts of 550 bases and 680 bases in Northern blots of RNA-isolated from anthers. RNA dot blots have shown that TA39-related transcripts accumulate and decay with the same temporal sequence as five other anther-specific transcripts, all of which are localized within the tapetum (Koltunow *et al.*, 1990).

Anthers of *Nicotinia tobacum* (cv Ky17) were collected at the tetrad stage and handled by standard cytological techniques

(Berlyn *et al.*, 1976). Anthers were dehydrated in t-butanol and embedded in paraffin, then sliced into 8 μm thick sections and fixed to slides. DNA fragments of clone TA39 and another cDNA clone (LA2: an epidermis-specific mRNA) were excised from plasmids, purified by gel electrophoresis and labeled by nick translation with biotin-14-dATP, using the BioNick Labeling System (BRL) according to directions of the manufacturer. *In situ* hybridization of fixed anther sections with biotin labeled probes was carried out and detected using the DNA Detection System of BRL. In this system, streptavidin binds biotinylated probe DNA and biotinylated alkaline phosphatase, resulting in precipitation of nitroblue tetrazolium in cells in which the probe hybridizes to target nucleic acids.

Examination of these *in situ* hybridization analyses showed that the anther locules of the tested specimens contained tetrad stage microspores. In anther sections probed with TA39 DNA, only the tetrads accumulated tetrazolium dye. In contrast, anther sections probed with a control DNA (LA2) accumulated dye in the epidermal layer. This tissue-specific control demonstrates that the observed precipitation of dye in microspores of anther sections probed by TA39 DNA is not due to nonspecific retention of DNA or detection system components by the microspores.

Example 3: Isolation of T39 Genomic Clones Comprising Sequences Homologous to Microspore-Specific mRNA; T39 Promoters

This example provides methods of isolation of genomic DNA clones comprising sequences homologous to any microspore-specific mRNA for which a nucleic acid probe is available. The approach described is useful for isolating microspore-specific regulatory sequences from any plant species which has microspore-specific mRNA that is homologous to such an available probe.

A tobacco anther-specific cDNA clone, TA39, was obtained from Dr. Robert Goldberg of UCLA. TA39 hybridizes to mRNA from anthers in a similar temporal pattern as seen with several tapetum-specific transcripts (Kultunow *et al.*, 1990).

In situ hybridizations showed that TA39 is present at low levels in microspores and connective tissue during stage -1 to +1 and then at higher levels in the tapetum from stage 1 through 6 (Goldberg *et al* 1993).

A genomic library of a selected plant, for instance a commercially available library of DNA fragment from *N. tabacum*, var. NK326 (Clontech Laboratories, Inc., Palo Alto, California; catalog FL1070D), partially digested with *MboI* and cloned into the plasmid EMBL-3, was screened for clones having homology to cDNA clone TA39. Standard hybridization methods were used, such as are described in Sambrook *et al.*, 1989. Candidate clones were purified by three or more cycles of picking plaques, replating, and reprobing with a TA39 cDNA insert, until consistently hybridizing plaques were either purified or shown not be present.

Two distinguishable families of genomic tobacco DNA clones related to the TA39 cDNA clone were identified, each represented by two overlapping clones within each family. One clone of each family was selected for detailed characterization, designated clones 8B3 (FIG. 24) and 14B1 (FIG. 25). The region of homology with TA39 in each of these genomic clones, as well as the regions immediately upstream and downstream of these regions of homology, were mapped by restriction enzyme cleavage analysis and DNA hybridization.

These coding sequences and associated 5' presumptive regulatory regions were isolated as subclones and then further subcloned for sequencing. Thus, nested sets of deletions of each genomic clone were produced by using exoIII and mung bean nucleases supplied in a kit by Stratagene. The nested deletions were sequenced by the dideoxy chain termination method of Sanger with an automated DNA sequencer (Applied Biosystems 373A) at the Nucleic Acids Facility of the Iowa State University. The cDNA insert of TA39 was also sequenced for comparison. Within the region of homology with the TA39 cDNA of a microspore-specific mRNA, genomic clone 8B3 is completely homologous with TA39, while the comparable portion of genomic clone 14B1 is about 90% homologous with TA39.

The starting points for transcription of the 14B1 and 8B3 genomic clones was mapped by primer extension experiments to a

single nucleotide, 83 bases upstream of the putative translational start site. A perfect TATA box appears 31 bp upstream of the mapped start of transcription in each clone, and a major open reading frame of 110 amino acids is intact downstream of the start of transcription in both clones (i.e., at the position designated "+83" relative to the transcription initiation site). Both clones also have a polyadenylation recognition site, 29 bp and 37 bp downstream of a translational stop codon in clones 14B1 and 8B3, respectively.

Example 4: Isolation of a DNA Segment Comprising a Microspore-Specific Genetic Control Sequence

Novel cDNA clones suitable for probes may be identified by screening of clones for hybridization with an oligonucleotide probe comprising a portion of a TA39 sequence, followed by sequencing of a hybridizing clone to determine the extent of identity of its complete sequence with that of cDNA clone TA39. For this purpose, stringent hybridization conditions are preferred. Although stringent hybridization may be performed for the purposes of the present invention according to a variety of conditions known in the art (*e.g.*, see J. Sambrook, 1989), one particular set of stringent hybridization conditions that has been used in the isolation of the exemplified control sequences comprises allowing about 12 hours of initial hybridization reaction at about 65°C in 6 x SSP, 0.1% SDS solution, followed by washing at about 65°C with 1 x SSC, 0.1% SDS solution and by washing at room temperature with 0.2 x SSC, 0.1% SDS solution.

For cDNA clones having only partial homology with clone TA39, such as a cDNA clone isolated from a plant other than tobacco, whether the mRNA from which the new cDNA is derived is a microspore-specific mRNA can be determined by temporal and, most precisely, by spatial analyses of expression of that mRNA in male flower tissues. See, for instance, analytical methods in Kultonow *et al.* (1990); Domon *et al* (1990), which disclose anther-specific cDNAs from sunflower (*Helianthus annuus*); Roberts, *et al.* (1991), which describes a *Brassica napus* mRNA said to be expressed specifically in developing microspores; Scott, *et al.* (1991), which describes cDNA libraries made from

developing anthers, including isolation of both tapetum-specific and microspore-specific cDNA clones; and Albani *et al.* (1990), which describes a pollen-specific gene family from *Brassica napus* which is activated during early microspore development.

The particular form of the probe for a microspore-specific mRNA related to clone TA39 encompasses any form of polynucleotide, a DNA or RNA molecule, single or double-stranded, having the physical properties needed to hybridize with a DNA molecule coding for the amino acid sequence encoded by TA39. General requirements for effective nucleic acid probes, including size, base composition, and extent of homology, are well known and described in the art. See, for instance, J. Sambrook *et al.*, (1989). However, a preferred probe, particularly when seeking DNA segments having nucleotide sequences that are not identical to the probe sequences, is a probe comprising the entire sequence of at least one strand of a cDNA derived from a microspore-specific mRNA. Such a full length probe provides a better opportunity to detect a partially homologous DNA molecule than does a probe containing only a portion of an mRNA-derived sequence. Thus, any portion of a given mRNA sequence might be less conserved in related microspore-specific genes than other parts of that mRNA sequence, whether these related genes derive from the same plant species from which the mRNA originated or from other species.

The method of isolating a DNA segment comprising a microspore-specific genetic control sequence further includes a step of isolating one or more fragments of genomic plant DNA that hybridize with the above nucleic acid probe under standard conditions for stringent hybridization. These fragments may be obtained from the same plant species from which the probe-related mRNA originated or from another species of angiosperm. Particular plants that are suitable for practice of this method of isolating microspore-specific gene control sequences of this invention include both monocots, particularly cereals such as corn, and dicots, for instance Canola and sunflower.

DNA fragments to be examined for microspore-specific control sequences typically are prepared by cloning in a vector suitable for such screening of large genomic DNA fragments

comprising open reading frames and associated regulatory sequences. For instance, preparation and hybridization screening of genomic plant DNA libraries may be performed for this purpose as described in any of: Albani, *et al.*, 1991, which describes genes from *Brassica napus*; Brown *et al.*, 1990, which discloses genes from *Oenothera organesis*; Guerrero, *et al.*, 1990, and Hamilton *et al.*, 1989, which disclose maize genes; or Twell, *et al.*, 1989, which describes an anther-specific gene from tomato. Alternatively, a commercially available library of plant genomic DNA clones may be obtained and screened.

The nucleotide sequence of any isolated fragment of plant genomic DNA that hybridizes with a probe according to this invention is established by standard DNA sequencing methods. Then this sequence is examined to determine whether it comprises a first DNA segment that hybridizes with the probe at a sequence of the first segment that encodes at least a 5'-end of a complete open reading frame. This 5'-end of an open reading frame is identified by a translational start codon followed by a sequence that contains an open reading frame and hybridizes with the probe of this method.

A genomic DNA fragment suitable for isolation of microspore-specific control sequences further includes a second DNA segment that comprises a sequence adjacent to the 5'-end of the complete open reading frame which hybridizes with the probe. In this context, a sequence that is "adjacent to the 5'-end of the complete open reading" includes any portion of a sequence that is upstream of this open reading frame in a genomic DNA fragment of this invention. In general, control sequences that regulate expression of an adjacent open reading frame may be located within a few hundred base pairs of this open reading frame; but, in some cases, certain control elements that are adjacent to an open reading frame may be located up to several kilobases away from that reading frame. In any case, by virtue of being adjacent to the microspore-specific mRNA-related sequences of a genomic DNA fragment of this invention, the sequences of the second segment of such a genomic DNA fragment constitutes presumptive microspore-specific control sequences.

The method of isolating a DNA segment comprising microspore-specific control sequences further comprises a step of testing each second DNA segment of a genomic DNA fragment for induction of microspore-specific expression of a DNA sequence that is operatively linked to the second DNA segment. This testing of segments containing presumptive microspore-specific control sequences serves to identify DNA segments comprising functional microspore-specific genetic control sequences that are suitable for engineering microspore-specific control of heterologous sequences in transgenic plants according to the present invention.

Testing of presumptive microspore-specific control sequences is accomplished by measuring expression of a convenient "reporter" sequence that is operatively linked to presumptive microspore-specific control sequences in microspores and other plant tissues. The general requirement for this reporter sequence is that the level of expression of this sequence can be readily ascertained in microspores and derivative tissues (e.g., pollen), as well as in other tissues (e.g., leaf) wherein microspore-specific control sequences should not induce expression of an operatively linked reporter sequence.

Example 5: Testing for Microspore-specific Expression of a Heterologous Gene that is Operatively Linked to Presumptive Control Sequences of Genomic DNA Clones

This example illustrates the use of microspore-specific regulatory regions from genomic DNA clones to provide microspore-specific control of expression of a heterologous reporter gene in a transient gene expression assay.

The putative promoters of 8B3 (FIG. 24) and 14B1 (FIG. 25) were each fused to an open reading frame of a reporter gene (*uidA*) encoding beta-glucuronidase (GUS), followed by the 3' untranslated region of the proteinase II (pinII) gene from potato. In one version, comprising a "translational" fusion, each promoter was cloned from the beginning of the available upstream sequences to the start of translation at nucleotide +83. In another variation designated a "transcriptional" fusion, each promoter was cloned from the beginning of available upstream sequences to just beyond the start of transcription, at

nucleotide +4. The latter constructs contained the non-translated leader of Tobacco Mosaic Virus (omega') between the promoter and *uidA* sequences. Translational gene fusions analogous to those containing the GUS reporter gene were also constructed for another model gene, the firefly luciferase coding region.

The *uidA* gene fusions were tested in transient expression assays on tobacco (cv. *Petite Havana)* stage 3-4 anther slices bombarded by a particle gun with DNA precipitated onto 1.8 μm tungsten beads. See, for instance, Twell *et al.*, 1989. Each shot contained 0.5 μg of DNA. Dark blue-staining spots were observed on anther slices and in individual microspores, indicating that transient expression of the GUS gene had occurred in microspores. The source of spots that were observed occasionally on the anther surface could not be distinguished as to whether they arose from anther cells or stray microspores. However, in additional tests with isolated microspores and leaves, transient expression was confirmed for *uidA* and luciferase gene fusions in microspores. Transient assays of the luciferase constructs in leaf pieces demonstrated that no gene expression activity of the microspore-specific control sequences was observed in leaves, using the most sensitive assay available (luciferase-catalyzed luminescence detection).

Example 6: Preparation of Genetic Constructs for Microspore-specific Expression of Genes for Insect Control or Male Sterility

This example illustrates genetic engineering methods for producing constructs that provide microspore-specific gene expression of heterologous genes, such as genes that effect insect control or male sterility, in transgenic plants.

To provide constructs for microspore-specific expression of genes encoding desired proteins, for instance, a selected insect-control gene or male sterility gene, a DNA segment comprising microspore-specific regulatory sequences of this invention is operatively linked to a heterologous gene, and to 3' non-translated sequences, as needed, for providing translational and transcriptional control appropriate for the selected

heterologous gene. The regulatory sequences are fused with heterologous gene sequences, for example, by modifying the beginning of the open reading frame of the heterologous gene to include a restriction enzyme cleavage site. Advantageously, this cleavage site is an *NcoI* site or another site compatible for ligation with an *NcoI* site, because the sequences of such sites comprise an ATG translation start codon.

A variety of genotypes were used for this example wherein xanthi tobacco transformations were performed at the 10 day germination stage.

The constructs are described as follows:

-- pPHI3621 (FIG. 4) + pPHI1285 (FIG. 6) [QM, maize sense + BAR]

-- pPHI3622 (FIG. 5) + pPHI1285 (FIG. 6) [QM, maize antisense + BAR]

-- pPHI4722 (FIG. 7) + pPHI1285 (FIG. 6) [QM, human sense + BAR]

-- pPHI4723 (FIG. 8) + pPHI1285 (FIG. 6) [QM, human sense + BAR]

-- pPHI265 + pPHI1285 [GUS + BAR]

-- pPHI1285 [BAR]

To achieve transformation, a particle gun bombardment was used, a GE Helium gun and 650PSI rupture disks. One bombardment was done per sample, for a total of 0.1 μg.

Tobacco was germinated and observed *in vitro* on 272 medium for 10-14 days before the following steps. One day before the experiment, cotyledons and first leaves were cut into halves and placed on sterile filters containing 1.5ml of 530 medium + 0.25M sorbitol. Incubation was done at 28°C in the dark overnight. Leaf material was dissected under liquid medium to prevent desiccation. Eight leaf sections per plate were cultured, 5 plates were prepared per QM transformation, and 3 plates were prepared per control transformation.

Following bombardment, all samples were maintained on the original filters for 2 days before transferring them to selection medium

After 48 hours, tissue was transferred to 526+Basta (526H) medium, leaving leaf tissue on the filters. Colony recovery generally occurred at 2-3 weeks post bombardment.

After 4 weeks, cotyledons/colonies were transferred to 528S medium. Plantlets from transformed colonies were cut off of the base callus and transferred to 272N medium to allow for root formation to occur. When roots were well established, plants were transferred to greenhouse for maturing.

The results were as follows:

126 colonies were recovered from all DNA treatments this study. PCR analysis was completed on 50 total colonies by randomly sampling 12 from each of the DNA treatments. Data from this analysis are shown below:

DNA Treatment	Percent PCR	Percent Plant Recovery
pPHI3621/pPHI1285	90%	14.3%
pPHI3622/pPHI1285	62.5%	20%
pPHI4722/pPHI1285	66.7%	16.6%
pPHI4723/pPHI1285	75%	28.6%
pPHI265/pPHI1285	100%	100%

Differences in growth rates were observed at 6 weeks post-bombardment. The observation most notable was that colonies recovered from transformations with pPHI3621/pPHI1285 and pPHI3622/1285 showed established colony death, especially from the pPHI3622/pPHI1285 treatment. No noticeable differences in growth were noted for the other transformations when compared to the control, pPHI265/pPHI1285 colonies.

Example 7: Stable BMS Transformation to Evaluate the Effect and Expression of QM Gene in Sense and Antisense Orientation and in the GRP/GRE Inducible Gene System

The genotype used was BMS P-38 in maize suspensions.

DNA constructs were:

pPHI4719 (FIG. 9) + pPHI1285 (FIG. 6) [35S-QM sense + 35S-BAR]

pPHI4720 (FIG. 10) + pPHI1285 (FIG. 6) [35S-QM antisense + 35S-BAR]

Particle gun bombardment was used (a GE helium gun, and 650PSI ruptured disks). One bombardment was done per sample.

One day after subculture, liquid was vacuumed off the cells and 2 grams of material was resuspended in 20ml 237 + 0.25M sorbitol medium. Cells were incubated at 28°C on shaker apparatus for 2-4 hours.

0.5ml of cells were plated onto double layers of Whatman filters moistened with 1.5ml 237 + 0.25M sorbitol medium. The cell density per plate was about 50mg.

6 samples were completed for each DNA treatment, including 2 samples as unshot controls.

Following bombardment, filters with cells were transferred to 115 medium and returned to the dark at 28°C for 48 hours.

Cells were transferred to 306E selection medium after 48 hours by scraping the cells off the filter, resuspending them in 2ml of 237 medium, and plating them in 1 ml per plate for each sample.

Colony recovery was monitored. When a colony was identified, it was separated from the others to maintain identity.

Induction assays may be performed after PCR analyses confirms presence of genes in transgenic colonies.

While colony recovery occurred from all transformations in this example, the majority of recovery came from the pPHI1285 positive control treatment. Data for colony recovery are shown below:

DNA Treatment	*N	Colonies Recovered
pPHI4719/pPHI1285	9	3
pPHI4720/pPHI1285	11	4
pPHI1285	11	44

*N denotes the number of samples bombarded per DNA treatment.

Both the 35S sense and antisense constructs for the QM gene were toxic to BMS colony recovery.

Example 8: Using a Maize Tapetum Specific Promoter for Transformation

Experiment Protocols

Repetition 1, 2, and 5:

Goal: Recover transgenic colonies, plants and progeny of maize resistant to Basta/Bialaphos and expressing GUS driven by the tapetum specific SGB6g1 promoter.

Genotype: 54-68-5 B1-1 (Repetition 1) or 54-68-5 161F3 (Repetition 2) 54-68-5 161F4 (Repetition 5)

Medium: 237 liquid suspension medium for maize
115, callus maintenance medium for maize
115E, callus selection medium containing 5mg/L Basta
115B, callus selection medium containing 3mg/L Bialaphos

Tissue Treatment:
-Sieve cells through 710um mesh one day after subculture
-Resuspend in 237+3% PEG at 50mg/ml plate density
-Incubate in 3% PEG overnight
-Plate cells, 0.5ml/plate onto glass filters 934-AH atop a Whatman filter moistened with 1ml 237+3% PEG medium
-Transfer cells on glass filter to 115 medium following bombardment

Particle gun bombardment:
DuPont helium gun (Repetitions 1 and 5)
650 PSI rupture disks (Repetitions 1 and 5)
DuPont PDS-1000 gun (Repetition 2)
0.230" stopping plates, Acetyl macroprojectiles (Repetition 2)
One bombardment per sample (Repetitions 1 and 5)
Two bombardments per sample (Repetition 2)
Pioneer tungsten modified DNA protocols, specific to each gun

DNA:
pPHI687 (FIG. 11) + pPHI610 (FIG. 12)

pPHI460 (FIG. 13) + pPHI610 (FIG. 12)
pPHI1952 (FIG. 14) + pPHI610 (FIG. 12)
pPHI2125 (FIG. 15) + pPHI610 (FIG. 12)

Treatment/Assay following bombardment:

-Look for R gene expression 24-48 hours post bombardment
-Transfer samples to 115E (repetitions 1) 48 hours post bombardment. Transfer samples to 115B (repetition 2 and 5) 7 days post bombardment
-Transfer cells off filters 2 weeks following transfer to selection
-PCR assay colonies for reporter gene prior to plant regeneration
-Maintain samples at 28°C in the dark

Repetition 1:

PCR assays were completed on 16 independent colonies recovered on 5mg/L Basta selection. One colony, #9 plate 1CZ, pPHI610+pPHI2125 was PCR positive for GUS (pPHI2125). All colonies were Type I phenotypes--however, the nonselected positive control also became a Type I phenotype. This phenotype tends to be common in the 54-68-5 B1-1 line. After 12 weeks on 5mg/L Basta selection, all PCR negative colonies were discarded along with all remaining nonembryogenic tissue. Colony 2 from Sample #9 plate 1 was transferred to 288E (Regeneration medium + 5mg/L Basta). Eight colonies remained to be PCR assayed for the presence of the GUS gene. Of these eight colonies, three were PCR positive for GUS from either the translational fusion (pPHI2125) or the transcriptional fusion (pPHI1952).

Repetition 5:

PCR assays were completed on nine independent colonies recovered on 3mg/L Bialaphos selection. All colonies were PCR positive for the GUS gene, indicating the presence of either pPHI2125 or pPHI1952. Gene controls used in this experiment (pPHI460) have yielded 9 stable transformants, all of which have areas that stain blue in a GUS cytochemical assay. Growth was much faster in the gene controls than in the transgenics recovered from the SGB6g1:GUS constructs.

After 12 weeks under selection pressure, only fast growing, embryogenic colonies were kept--all other material was discarded. Colonies testing PCR positive were transferred to regeneration medium for plant recovery. Basta enzyme assays were completed on a portion of the colonies. Results shown do not indicate a high degree of transgenics actively showing resistance to Basta. From previous work and other researchers' experiences with this assay, a more reliable measure of transformation is determining whether the cell morphology of the recovered colonies closely resembles that of the nonselected controls, and comparing the rate of growth the recovered colonies exhibit.

Example 9: Construction of Plasmids Containing The Maize QM Gene

Plasmid pPHI3621 (FIGURE 4) which expresses the QM gene in the sense orientation was constructed using pPHI1527 as one parent. pPHI1527 (FIGURE 16) contains the plasmid pUC18 as the backbone (Yanisch-Perron *et al.*, 1985) which contains the restriction sites necessary for cloning and the ampicillin resistance gene as a selectable marker. It also contains the cauliflower mosaic virus (CaMV) 35S promoter and enhancer sequences (Gardner, *et al.* 1981) the tobacco mosaic virus leader sequences, O' (Gallie *et al.*, 1987), the firefly luciferase reporter gene (Ow, *et al.* 1986) and the PinII transcription terminator sequences (Hynheung, *et al.* 1989).

The second parent of pPHI3621 was pPHI3620, which contained the maize QM gene in pBluescript KS (FIGURE 17). pPHI3621 was generated by digestion of both pPHI3620 and pPHI1527 with NcoI and KpnI and isolation of the insert band from pPHI3620 and the larger plasmid band from pPHI1527 on low melting point (LMP) agarose gels. This strategy replaced the luciferase gene with the maize QM gene. The bands were pooled and ligated to form pPHI3621.

pPHI3622 (FIGURE 5), which expresses the antisense of the maize QM gene was also constructed using pPHI1527 and pPHI3620 as parents, but by digestion of both with SalI and SacI. The insert band from pPHI3620 and the larger plasmid band from pPHI1527 were isolated from LMP agarose gels; the fragments were pooled and

ligated. Again, this procedure replaced the luciferase gene with the maize QM gene in an antisense orientation.

Tissue specific expression vectors were constructed in the same manner except that the CaMV constitutive promoter was replaced with the TA39 anther specific promoters, 14B1 and 8B3 (Garnaat *et al.*, 1991). pPHI1493 (FIGURE 18) containing the 14B1 promoter was digested with NcoI and NsiI as was pPHI3621 (parent 2). The small insert band from pPHI3621 and the larger plasmid band were isolated by LMP agarose gel electrophoresis, were pooled and ligated. This yielded pPHI4745 (FIGURE 19) which contained the maize QM gene in the sense orientation with the 14B1 promoter. The maize QM antisense construct was made by digestion of pPHI4745 with SmaI and NsiI and digestion of pPHI3622 with SalI (which was filled in with Klenow fragment) and NsiI. The large plasmid band from pPHI4745 and the insert band from pPHI3622 were isolated by LMP gel, pooled and ligated. This yielded the plasmid L62 (TA39 14B1) (FIGURE 20).

The expression vectors containing the 8B3 anther specific promoter were constructed by digestion of pPHI4855 (FIGURE 21) with BamHI and NotI. pPHI4855 contained all of the above described sequences with the additional sequences encoding the ß-glucuronidase gene (Walden and Schell, 1990). The other parent, pPHI4745 was also digested with BamHI and NotI. The large plasmid band from the pPHI4855 and the insert band from pPHI4745 were purified from LMP agarose, pooled and ligated. The resulting plasmid, L59 (FIGURE 22) contained the maize QM in the sense orientation driven by the anther specific promoter 8B3. The antisense construct was made by digestion of pPHI4855 with SmaI and NsiI and pPHI3622 with SalI (then filled in with Klenow fragment) and NsiI. The large plasmid band from pPHI4855 and the insert band from pPHI3622 were isolated from LMP agarose gel, pooled and ligated. This gave L61 (FIGURE 23) the antisense orientation of the maize QM gene under control of the 8B3 promoter.

Example 10: Production of Male Sterile Tobacco Plants by Transformation with a QM Gene, Sense and Antisense

Tobacco leaf explants were co-bombarded with 35S::BAR (pPHI1285) and one of the following plasmids:

L59:	TA39(8B3):QM
L61:	TA39(8B3):antisense QM
L62:	TA39(14B1):antisense QM
4745:	TA39(14B1):QM

A BioRad helium biolistics gun (DuPont) was used to bombard the tobacco leaf explants. The BAR gene was used as a selectable marker to determine which cells received the plasmids.

A tobacco anther promoter (TA39) was used in all four plasmids. This is a tapetum specific promoter. "8B3" and "14B1" were isolates of TA39.

The L59 plasmid (FIGURE 22) is identical to the L61 plasmid (FIGURE 23) except that the L59 plasmid included a QM sense gene from maize, and the L61 plasmid included an antisense gene to the maize QM gene.

The L62 plasmid (FIGURE 20) is identical to the pPHI4745 gene except that the pPHI4745 plasmid included a QM sense gene from maize, and the L62 plasmid included an antisense gene to the maize QM gene.

A leaf punch was made in sections of the explant that showed incorporation of the BAR gene. The polymerase chain reaction (Perkin-Elmer), well-known to those of skill in the art, was used to amplify the maize QM gene incorporated in the tobacco cells.

PCR+ cells were selected from bialophos resistant calli and used to regenerate tobacco plants (See Example 1).

Fertility of the regenerated plants was determined by checking for 1) pollen shed; and the ability to self-fertilize. In all cases of male sterile plants shown below, both criteria were met. As can be seen, male sterile plants were from cells transformed with L62 and from cells transformed with pPHI4745. No tobacco plants were produced from cells transformed with L59, and only two plants were produced that were transformed with L61. Experimental failure due to a low frequency of successful

bombardment, may explain the lack of male fertile plants associated with those two plasmids.

Male sterile tobacco plants were associated with the presence both of the maize QM gene and of the antisense to the maize QM gene. A likely explanation is that a balance of QM gene expression is required for normal development. In yeast, the QM balance appears to be essential for normal development.

In the presence of the maize QM gene, too much QM expression may occur, an expression product from the corn gene may be a disruptive mutant in a tobacco cell, or the maize QM gene may be expressed at an inappropriate time in development.

The maize QM antisense gene product may bind to the tobacco QM gene, turning it off at a crucial time in development.

Analysis of the QM nucleotide sequences of the tobacco and the corn gene suggests there may be sufficient homology that the antisense product directed to one gene, may also bind the other.

Fertility of Plants Regenerated from Bialophos Resistant Callus

Plasmid	Total Number of Plants	Number of Male Sterile L59 Plants
L61	2	0
L62	9	3
pPHI4745	12	6

Materials and Methods

USE OF QM GENE IN SENSE ORIENTATION: The nucleotide segment of the QM gene isolated from maize or other plant sources is fused at its upstream (5') end to a promoter which allows expression of the sense strand in a particular target plant cell and is fused at its downstream (3') end to suitable transcription terminator and polyadenylation signals known to function in that cell. Preferred promoters include those that are known to direct expression in the desired target cell, which includes "constitutive" promoters such as 35S from CaMV and the promoter from the ubiquitin gene that are known to direct expression in a wide variety of plant cell types. 35S is likely to direct expression in both monocots such as corn and dicots such as tobacco and canola. However, the ubiquitin promoter for tobacco preferably is derived from a dicot source. The ubiquitin promoter for use in monocots such as corn preferably is derived from a monocot source. Other suitable promoters include those which are known to be inducible under specific conditions, such as in response to particular chemical treatments for example, an herbicide.

Terminator/polyadenylation signals include those that are known to function in the target cell of interest. Preferred are signals from genes such as pinl1 (proteinase inhibitor II from potato) or T-DNA genes such as OCS or NOS, which are known to function in a wide variety of plant cell types, including those of dicots and monocots such as corn. When the target cell is from a monocot like corn, it is preferred, but not necessarily required, that an intron from a monocot gene be inserted between the promoter and the QM gene. Examples would be an intron (such as intron 1 or 6) from the Adh1 gene of corn.

In an illustrative embodiment, the nucleotide segment of the QM gene is fused at its upstream (5') end to a promoter which is known to be specific for, or show a strong preference for expression in, a tissue or cell that is critical for pollen development. The anther is an example of such a tissue. A tapetal cell or developing microspore is an example of a suitable cell. The segment is fused at its downstream (3') end to suitable transcription terminator and polyadenylation signals

also known to function in that cell. Preferred promoters would be SGB6 for maize and TA39 (from tobacco) and the promoter Bp4A of clone L4 (from *B. napus*, WO 90/08828) for dicots.

USE OF QM GENE IN ANTISENSE ORIENTATION: The antisense form of the QM gene is fused at its upstream (5') end to a promoter which directs expression in a particular target plant cell, and is fused at its downstream (3') end to suitable transcription terminator and polyadenylation signals also known to function in that cell. An embodiment of a target cell in this case is a cell in which the QM gene or a gene highly homologous to the QM gene is known to be expressed so that the antisense works effectively. Preferred promoters encompass those that are known to direct expression in the desired target cell, suitable candidates include "constitutive" promoters such as 35S from CaMV and the promoter from the ubiquitin gene that are known to direct expression in a wide variety of plant cell types. 35S is expected to express in both monocots such as corn and dicots such as tobacco and canola. However, the ubiquitin promoter for tobacco is preferably from a dicot source, and the ubiquitin promoter for use in monocots such as corn is preferably from a monocot source. Other preferred promoters include those which are known to be inducible under specific conditions, such as in response to a particular chemical treatment for example, a herbicide. It is preferred that the antisense construct include the entire QM gene or at least several hundred nucleotides from the 5' end of the gene.

The nucleotide segment of the antisense form of the QM gene is fused at its upstream (5') end to a promoter which is known to be specific for, or show a strong preference for expression in, a tissue or cell critical for pollen development. An example of a suitable tissue is the anther. An example of a suitable cell is a tapetal cell or a developing microspore. The segment is fused at its downstream (3') end to suitable transcription terminator and polyadenylation signals also known to function in the cell or tissue. The target cell is a cell in which the QM gene or a gene highly homologous to the QM gene is known to direct expression so that the antisense works effectively.

TRANSFORMATION METHODS: Transformation methods for dicots include a number of different well-known methods for direct DNA delivery. Preferred is particle biolistics bombardment of leaf explants. Other methods include *Agrobacterium* delivery to explants; *Agrobacterium* cocultivation of protoplasts; electroporation; PEG uptake or other direct DNA delivery into protoplasts and the like. A preferred method for monocots such as corn is delivery of DNA to the treated cells by bombardment, but other methods such as electroporation can also be used.

Cells of a plant are transformed with the foreign DNA sequence of this invention in a conventional manner. If the plant to be transformed is susceptible to *Agrobacterium* infections, it is preferred to use a vector containing the foreign DNA sequence, which is a disarmed Ti-plasmid. The transformation can be carried out using procedures described, for example, in EP 0 116 718 and EP 0 270 822. Preferred Ti-plasmid vectors contain the foreign DNA sequence between the border sequences, or at least located upstream of the right border sequence. Other types of vectors can be used for transforming the plant cell, using procedures such as direct gene transfer (see, for instance, EP 0 237 356, PCT publication WO/85/01856 and EP 0 275 069); *in vitro* protoplast transformation as described, for example, in U.S. patent No. 4,684,611; plant virus-mediated transformation as taught in EP 0 067 553 and U.S. patent No. 4,407,956, for example; and liposome-mediated transformation as described in U.S. patent No. 4,536,475, among others.

If the plant to be transformed is corn, recently developed transformation methods are suitable such as the methods described for certain lines of corn by Fromm *et al.*, 1990, and Gordon-Kamm *et al.*, 1990.

If the plant to be transformed is rice, recently developed transformation methods can be used such as the methods described for certain lines of rice by Shimamoto *et al.*, 1990, Datta *et al.*, 1990, Christou *et al.*, 1991, and Lee *et al.*, 1991.

If the plant to be transformed is wheat, a method analogous to those described above for corn or rice can be used. Preferably for the transformation of a monocotyledonous plant, particularly a cereal such as rice, corn or wheat, a method of

direct DNA transfer, such as a method of biolistic transformation or electroporation, is used. When using such a direct transfer method, it is preferred to minimize the DNA that is transferred so that essentially only the DNA sequence of this invention, the QM maize gene and associated regulatory regions, is integrated into the plant genome. In this regard, when a DNA sequence of this invention is constructed and multiplied in a plasmid in a bacterial host organism, it is preferred that, prior to transformation of a plant with the DNA sequence, plasmid sequences that are required for propagation in the bacterial host organism, such as on origin of replication, an antibiotic resistance gene for selection of the host organism, and the like, be separated from the parts of the plasmid that contain the foreign DNA sequence.

<u>TUNGSTEN/DNA PROTOCOL FOR DuPONT HELIUM GUN (PARTICLE BIOLISTIC BOMBARDMENT METHOD OF TRANSFORMATION)</u>

Weigh 60 mg 1.8 μm tungsten: put into 15ml centrifuge tube
Add 2ml 0.1M HnO_3: Sonicate on ice for 20 minutes
Withdraw HNO_3: Add 1 ml sterile deionized water and transfer sample to a 2ml Sarstedt tube. Sonicate briefly
Centrifuge to pellet particles
Withdraw H_2O: Add 1ml 100% EtOH - Sonicate briefly
Centrifuge to pellet particles
Withdraw H_2O: Add 1ml 100% EtOH - Sonicate briefly
Centrifuge to pellet particles
Withdraw EtOH. Add 1ml sterile deionized water. Sonicate.
Pipet 250μl of suspension into 4, 2ml tubes.
Add 750μl of sterile deionized H_2O to each tube.
Freeze tungsten sample between use.
Pipet 50μl tungsten/H_2O suspension into 1.5ml tube (Sonicate first)
Add 10μg DNA, Mix
Add 50μl 2.5M $CaCl_2$. Mix
Add 20μl 0.1M Spermidine. Mix
Sonicate briefly. Centrifuge for 10 seconds at 10,000 RPM.
Withdraw supernatant. Add 250μl 100% EtOH. Sonicate briefly.
Centrifuge at 10,000 RPM for 10 seconds
Withdraw supernatant. Add 60μl 100% EtOH.

PROTOCOL FOR CORN TRANSFORMATION TO RECOVER STABLE TRANSGENIC PLANTS

Day - 1 Cells are placed in liquid media and sieved (710um). 100-200 mg of cells are collected on 5.5 cm glass fiber filter over an area of 3.5 cm. Cells are transferred to media and incubated overnight.

Day - 8 Filter and cells are removed from media, dried and bombarded. Filter and cells are placed back on media.

Day - 5 Cells on the filter are transferred to selection media (3 mg bialophos).

Day - 12 Cells on the filter are transferred to fresh selection media.

Day - 19 Cells are scraped from the filter and dispersed in 5 ml of selection media containing 8.6% low melting point sea agarose. Cells and media ate spread over the surface of two 100mm x 15mm plates containing 20 ml of gel-rite solidified media.

Day - 40 Putative transformants are picked from plate.

Day - 61 Plates are checked for new colonies.

RNA ANALYSIS:

Total cellular RNA was prepared from B73 seedlings seven days following planting by the protocol of Chomczynski and Sacchi (1987). Poly (A)+ RNA was purified from leaf homogenates using the PolyAtract 1000 system (Promega). Northern blots were done as previously described (Thomas, 1980).

CHARACTERIZATION OF FLANKING REGIONS

Primer extension mapping of the RNA 5' termini followed the method of McKnight (1982). Oligonucleotides used for primer extension reactions were 5' and labeled 32-mer and 44-mers homologous to the TA39 cDNA from the last nucleotide of the start codon to 31 nucleotides and from 79 and 122 nucleotides downstream of the start codon, respectively. RNA was isolated from tobacco anthers using the guinidinum isothiocyante procedure of Chomcynski and Sacchi (1987) and purified using an oligo dT column.

PLASMIDS

Site directed mutagenesis (Su and El-Gewely, 1988) was used to create either a NcoI site at the start codon with the

oligonucleotide 5'CTAATTCCACCATGGCTTTTCTTGC3' or a PstI site 5 bases downstream of the putative start of transcription with the oligonucleotide 5'GTTTATGTTTTCGTATCTGCAGCTTGAAAAGATATTATATC3'.

For uidA reporter constructs, 5' flanking regions were fused at the NcoI site to the uidA reading frame with a 3' transcript processing signal from the protease inhibitor gene of potato (PI-II), or fused at the PstI site to the TMV untranslated leader Ω', uidA reading frame and PI-II. The uidA reporter constructs with Ω' were inserted into the Ti binary vector pALLTKRep. pALLTKRep differs from pBI101.1 (Jefferson, 1987) in that the CaMC 35S promoter driving the NPTII selectable marker instead of the nopaline synthase promoter. The plasmid pLAT52-7 which contains the tomato pollen-specific promoter and the uidA reporter gene, was kindly provided by Dr. Sheila McCormick of the USDA-ARS Plant Gene Expression Center, Albany, CA.

The lcf reporter plasmids were created by fusing 5' flanking regions of LAT52 or the TA39 genomic clones at the NcoI site to the firefly luciferase gene with PI-II 3'.

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U.S. patent No. 4,536,475
U.S. patent No. 4,684,611

WHAT IS CLAIMED IS:

1. An isolated polynucleotide molecule that encodes a developmental protein in a plant and that is capable of hybridizing to a mammalian QM gene.

2. A cDNA that consists essentially of 800-950 nucleotides, including a single open reading frame for a polypeptide that is homologous to the QM protein in mammals.

3. The cDNA of claim 2, wherein said open reading frame comprises three coding regions that respectively display a high degree of homology to corresponding regions which are characteristic of mammalian QM genes.

4. The cDNA of claim 2, wherein said polypeptide displays at least 50% homology with a mammalian in QM protein.

5. The cDNA of claim 3, wherein said conserved regions comprise nucleotide sequences at positions 30 to 100, 210 to 250, and 330-400, respectively, in relation to the N-terminal end of said cDNA.

6. The cDNA of claim 2, wherein the sequence of said nucleotides is according to FIGURE 1.

7. An isolated and purified protein having an estimated molecular weight of approximately 25 kD, an amino acid sequence which has a homology to the amino acid sequence of the QM protein in humans, a first, second and a third conserved amino acid sequence region, and a PI of approximately 11.0.

8. The protein of claim 7, wherein the first conserved region is a sequence including the first 10 amino acids of the amino terminus, the second conserved region is an amino acid sequence including approximately residues 51 to 60, said sequence forming an amphipathic helix and the third region includes residues 98-135.

9. The protein of claim 7, further defined as characterized by the amino acid sequence according to FIGURE 2.

10. A recombinant DNA molecule comprising the cDNA of claim 2 and a promoter capable of regulating transcription of said DNA in a plant cell.

11. The recombinant DNA molecule of claim 10, wherein the promoter is a tissue-specific promoter.

12. The recombinant DNA molecule of claim 11, wherein the promoter is the QM maize promoter.

13. The recombinant DNA molecule of claim 10, wherein the promoter is a cell-specific promoter.

14. The recombinant DNA molecule of claim 11 or 12, wherein the tissue or cell is crucial to the production of pollen.

15. The recombinant DNA molecule of claim 11, wherein the tissue is anther tissue.

16. The recombinant DNA molecule of claims 12, wherein said cell-specific promoter is expressed in a tapetal cell.

17. The recombinant DNA molecule of claim 10, wherein the promoter causes overexpression of the cDNA.

18. The recombinant DNA molecule of claim 10, wherein the promoter is an inducible promoter.

19. An isolated DNA molecule, the RNA transcript of which is complementary to the transcript of a cDNA according to claim 2.

20. A method of producing a male sterile plant, said method comprising transforming a plant cell with the recombinant molecule of claim 10.

21. A method of altering the normal development of a plant comprising transforming the plant with the recombinant molecule of claim 10.

22. A transgenic plant including the recombinant molecule of claim 10.

23. A promoter isolated from maize that controls the expression of the QM gene in plants.

24. The promoter of claim 23, according to the nucleotide sequence of FIGURE 3.

FIG. 1A(1)

```
GGATCCGCCG ACACCGACTG CCTACCTCAG CTGCCGTCGC C ATG GGC AGA AGG
                                        Met Gly Arg Arg
                                         1

CCT GCT AGA TGC TAT CGC CAG ATC AAG AAC AAG CCG TGC CCT AAG TCC
Pro Ala Arg Cys Tyr Arg Gln Ile Lys Asn Lys Pro Cys Pro Lys Ser
  5                 10                  15                  20

AGG TAC TGC CGT GGT GTC CCT GAC CCC AAG ATC AGG ATC TAC GAT GTC
Arg Tyr Cys Arg Gly Val Pro Asp Pro Lys Ile Arg Ile Tyr Asp Val
                 25                  30                  35

GGG ATG AAG AGG AAG GGT GTT GAT GAG TTC CCC TAC TGT GTG CAC CTT
Gly Met Lys Arg Lys Gly Val Asp Glu Phe Pro Tyr Cys Val His Leu
             40                  45                  50

GTC TCT TGG GAG AGG GAG AAT GTC TCC AGT GAG GCG CTC GAG GCT GCC
Val Ser Trp Glu Arg Glu Asn Val Ser Ser Glu Ala Leu Glu Ala Ala
         55                  60                  65

CGC ATT GTC TGT AAC AAG TAC ATG ACC AAG TCT GCA GGA AAG GAT GCC
Arg Ile Val Cys Asn Lys Tyr Met Thr Lys Ser Ala Gly Lys Asp Ala
     70                  75                  80

TTC CAC CTT AGG GTC CGG GTT CAC CCG TTC CAT GTC CTC CGT ATC AAC
Phe His Leu Arg Val Arg Val His Pro Phe His Val Leu Arg Ile Asn
 85                  90                  95                 100

AAG ATG CTT TCC TGT GCC GGG GCT GAT AGG CTC CAG ACT GGA ATG AGG
Lys Met Leu Ser Cys Ala Gly Ala Asp Arg Leu Gln Thr Gly Met Arg
                105                 110                 115

GGT GCC TTT GGC AAG CCT CAG GGC ACC TGT GCT AGG GTG GAC ATT GGT
Gly Ala Phe Gly Lys Pro Gln Gly Thr Cys Ala Arg Val Asp Ile Gly
            120                 125                 130
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FIG. 1A(2)

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CAG GTC CTC CTT TCC GTG CGC TGC AAG GAC AAC AAT GCT GCC CAT GCC
Gln Val Leu Leu Ser Val Arg Cys Lys Asp Asn Asn Ala Ala His Ala
      135                 140                 145

AGC GAA GCT CTG CGT CGC GCT AAG TTC AAG TTC CCT GCC CGC CAG AAG
Ser Glu Ala Leu Arg Arg Ala Lys Phe Lys Phe Pro Ala Arg Gln Lys
    150                 155                 160

ATC ATT GAG AGC AGA AAG TGG GGC TTC ACC AAG TTC AGC CGC GCT GAC
Ile Ile Glu Ser Arg Lys Trp Gly Phe Thr Lys Phe Ser Arg Ala Asp
165                 170                 175                 180

TAC CTG AAG TAC AAG AGC GAG GGC AGA ATT GTT CCT GAT GGT GTC AAC
Tyr Leu Lys Tyr Lys Ser Glu Gly Arg Ile Val Pro Asp Gly Val Asn
                185                 190                 195

GCA AAG CTG CTC GCC AAC CAC GGC AGA CTT GAG AAG CGT GCT CCT GGG
Ala Lys Leu Leu Ala Asn His Gly Arg Leu Glu Lys Arg Ala Pro Gly
            200                 205                 210

AAG GCT TTC CTC GAT GCC GTT GCT TAAGTGCGGA TGCGAATCCT GACGTTTTGC
Lys Ala Phe Leu Asp Ala Val Ala
        215                 220

TTTAGCGTAT CTTACTTTGC TTCGTGGAAC ATGAATTTCA AGTGTTTTGA GGGTATTACA

GTGCCTTATG TGAACTTGCC TATCTTGTGC TGAACATCGG AATGTATCCT CCGAGTATGT

TTAATCGCAT TAATTTTATT GGGAAATTGG TTGCGGAACA ATGTCCAATT TAACTCGAAT

TTGATTTCAA CACGGTCTTT TCTTT
```

FIG. 1B

```
Met Gly Arg Arg Pro Ala Arg Cys Tyr Arg Gln Ile Lys Asn Lys Pro
 1               5                  10                  15

Cys Pro Lys Ser Arg Tyr Cys Arg Gly Val Pro Asp Pro Lys Ile Arg
            20                  25                  30

Ile Tyr Asp Val Gly Met Lys Arg Lys Gly Val Asp Glu Phe Pro Tyr
        35                  40                  45

Cys Val His Leu Val Ser Trp Glu Arg Glu Asn Val Ser Ser Glu Ala
    50                  55                  60

Leu Glu Ala Ala Arg Ile Val Cys Asn Lys Tyr Met Thr Lys Ser Ala
65                  70                  75                  80

Gly Lys Asp Ala Phe His Leu Arg Val Arg Val His Pro Phe His Val
                85                  90                  95

Leu Arg Ile Asn Lys Met Leu Ser Cys Ala Gly Ala Asp Arg Leu Gln
            100                 105                 110

Thr Gly Met Arg Gly Ala Phe Gly Lys Pro Gln Gly Thr Cys Ala Arg
        115                 120                 125

Val Asp Ile Gly Gln Val Leu Leu Ser Val Arg Cys Lys Asp Asn Asn
    130                 135                 140

Ala Ala His Ala Ser Glu Ala Leu Arg Arg Ala Lys Phe Lys Phe Pro
145                 150                 155                 160

Ala Arg Gln Lys Ile Ile Glu Ser Arg Lys Trp Gly Phe Thr Lys Phe
                165                 170                 175

Ser Arg Ala Asp Tyr Leu Lys Tyr Lys Ser Glu Gly Arg Ile Val Pro
            180                 185                 190

Asp Gly Val Asn Ala Lys Leu Leu Ala Asn His Gly Arg Leu Glu Lys
        195                 200                 205

Arg Ala Pro Gly Lys Ala Phe Leu Asp Ala Val Ala
    210                 215                 220
```

FIG. 2

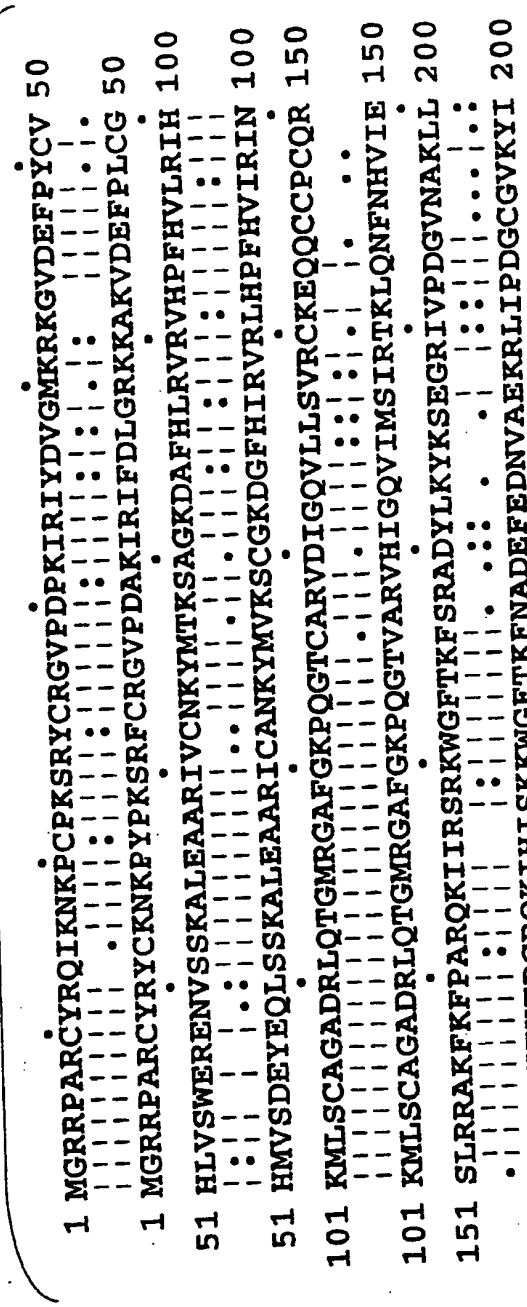

1 MGRRPARCYRQIKNKPCPKSRYCRGVPDPKIRIYDVGMKRKGVDEFPYCV 50
1 MGRRPARCYRYCKNKPYPKSRFCRGVPDAKIRIFDLGRKKAKVDEFPLCG 50
51 HLVSWERENVSSKALEAARIVCNKYMTKSAGKDAFHLRVRVHPFHVLRIH 100
51 HMVSDEYEQLSSKALEAARICANKYMVKSCGKDGFHIRVRLHPFHVIRIN 100
101 KMLSCAGADRLQTGMRGAFGKPQGTCARVDIGQVLLSVRCKEQQCCPCQR 150
101 KMLSCAGADRLQTGMRGAFGKPQGTVARVHIGQVIMSIRTKLQNFNHVIE 150
151 SLRRAKFKFPARQKIIRSRKWGFTKFSRADYLKYKSEGRIVPDGVNAKLL 200
151 ALRRAKFKFPGRQKIHISKKWGFTKFNADEFEDNVAEKRLIPDGCGVKYI 200
201 ANHGRLEKRAPGKAFLDAVA 220
201 PSRGPLDKWRALSS..... 214

FIG. 3

GCCGGCGCCGA CCGTTGAAAG
CTCCGCGAGC TTCTCCACCA GACCAGACGA CGAGCCCGCG NCGTCCGACA
TGTCACGCGC GGGACTCTCT AGGTTTCGTT GTGAGGGGGC GAGCGCGGTG
AGTGTGGACT GTGGAGGCGC AGGTGTGATG GTAGTAGAGT GGTAGGGTTT
TAGGGCAGCT CGTGGTGGCG CGGAGGAAAG GAAAACCGTC GTAGCCGACA
AGCCATCTGA TCCCCACGGC CACGGCATCA ACATGGGCTT GACCTTTTTG
GCCCGTGAGA ACTTAACAAC CCTGTTTGAG TGTTTTGATG TGGACAACGT
GGGCTCAGCC CAACACGGCC CTGCTTATTG ATTTTTTTCA GTGTCCAGCT
CGAGTGGCCG AGTGCCGGGG AGAGGAGGCG GCGGCGGCAA GGGCGCGACG
GGGGAGGATG ATGTCGCTGC AGCCTGCAGG AGTTGTGTTC TCTGGCCTTG
AGAGAAAGGA GTCGCCAATT TTAGTGGGCT CCTGTTGTGT TTTTCGGTCC
ATTCCTCCCA AGGCCCAGCA TCTGCCAGTC GCATGCCGTG ACGCACAAAA
CCCACGGCGG CACGGCTCCA TTCCGCGTCC GCACTCTCTA TATAAAGTGT
CCCTCTCTTC CCTCCAAGCC CTAGACGCAC CCCTTCCTCG TTTCGCCGCC
TCCGCCGACA CCGACTGCCT ACCTCAGCTG CCGTCGCCAT GGGCAGAAGT
AAGTTCGCCT TTTTGATTAA CCTCCTTCGT ACGCTTCCTA CTGCGTTGAT
TGTTGCAGTC CATAGGGTAC TTTCTTTTAG TCCGGCGCAA ATTTCGACTA
GATCCACACG AAATCGGGTA TATGCTTTTG ATGATCCCGG GGTTCTTACT
GTCCACGGGC TTAATATTTG ATGTTTGTTT GCTGAGTTGA TGATTTGTGG
AACTCGCAAA GTTGTTACGT ATTATCGTAT AAAGAAGAGA ATGGCTGGGA
CCTGGGGGAT TTGGTACCAT ATATTCACAT TTCCTGTGTA TAAAGTCAAC
ACGCTCATAA TTTAGAATCC GTGTAGACCT TTATCTGTCA AAAAAGGGGA
GTATTAGTCA TATTCCATGG
NcoI

FIG. 4

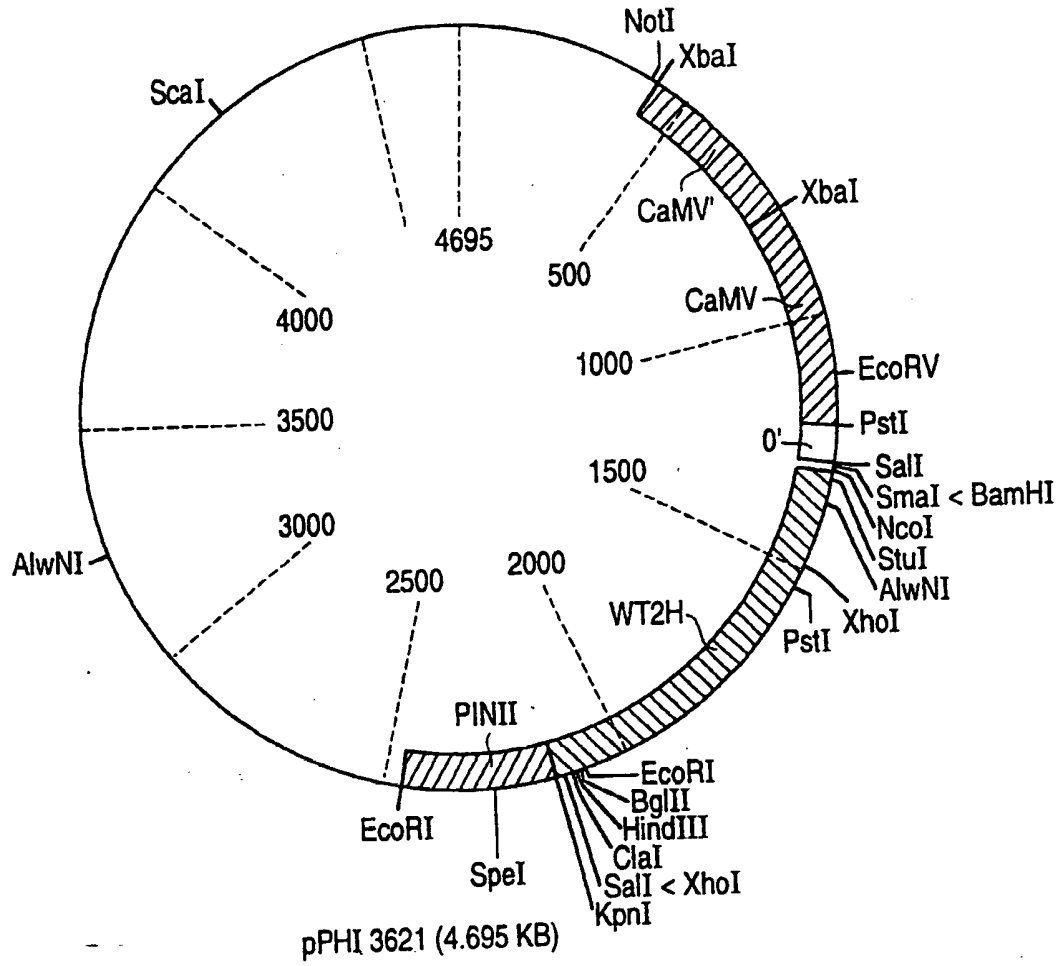
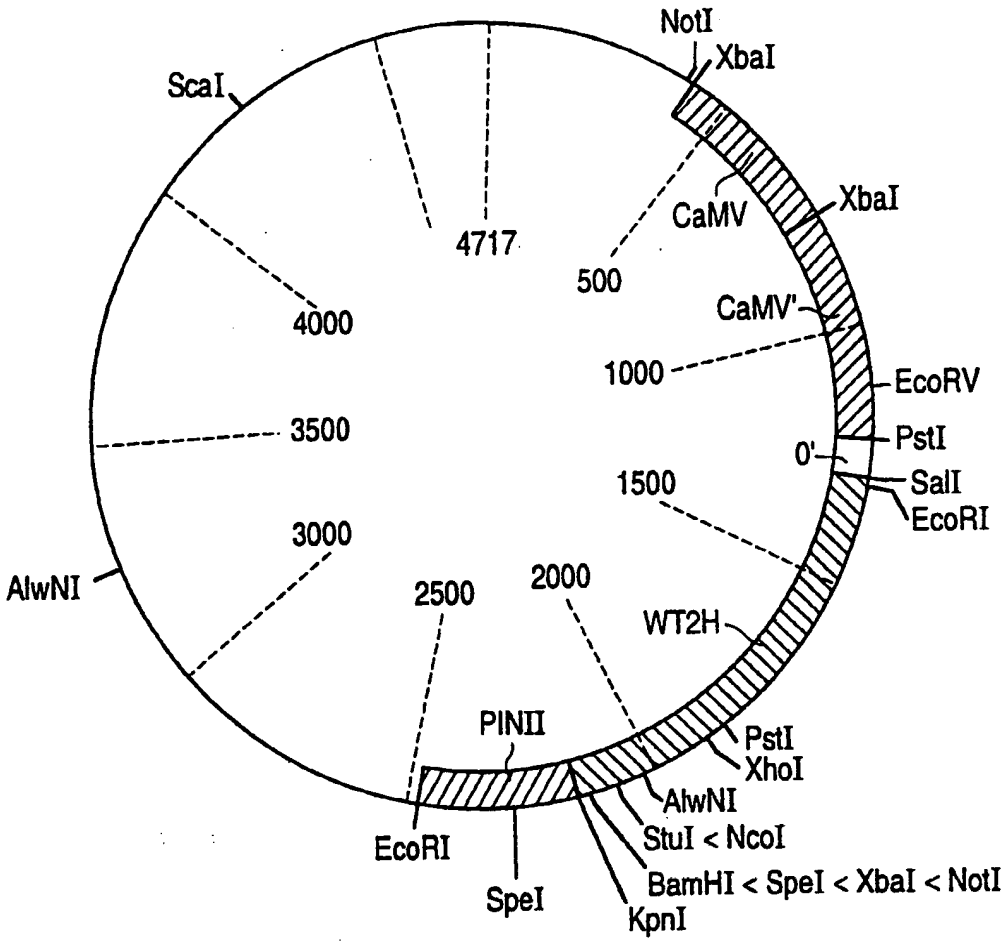
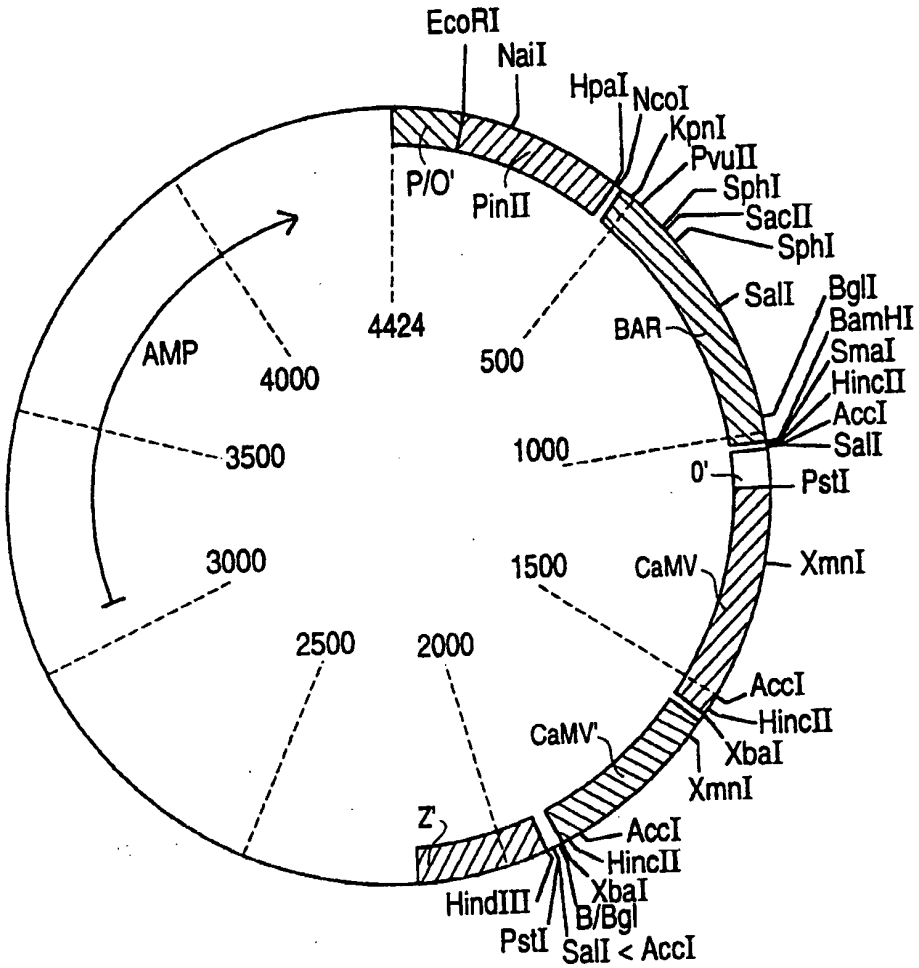

NotI
XbaI
ScaI
XbaI
CaMV'
4695
500
CaMV
4000
1000
EcoRV
3500
PstI
0'
SalI
1500
SmaI < BamHI
NcoI
3000
StuI
AlwNI
AlwNI
2500
2000
WT2H
XhoI
PstI
PINII
EcoRI
BglII
HindIII
EcoRI
ClaI
SpeI
SalI < XhoI
KpnI
pPHI 3621 (4.695 KB)

FIG. 5



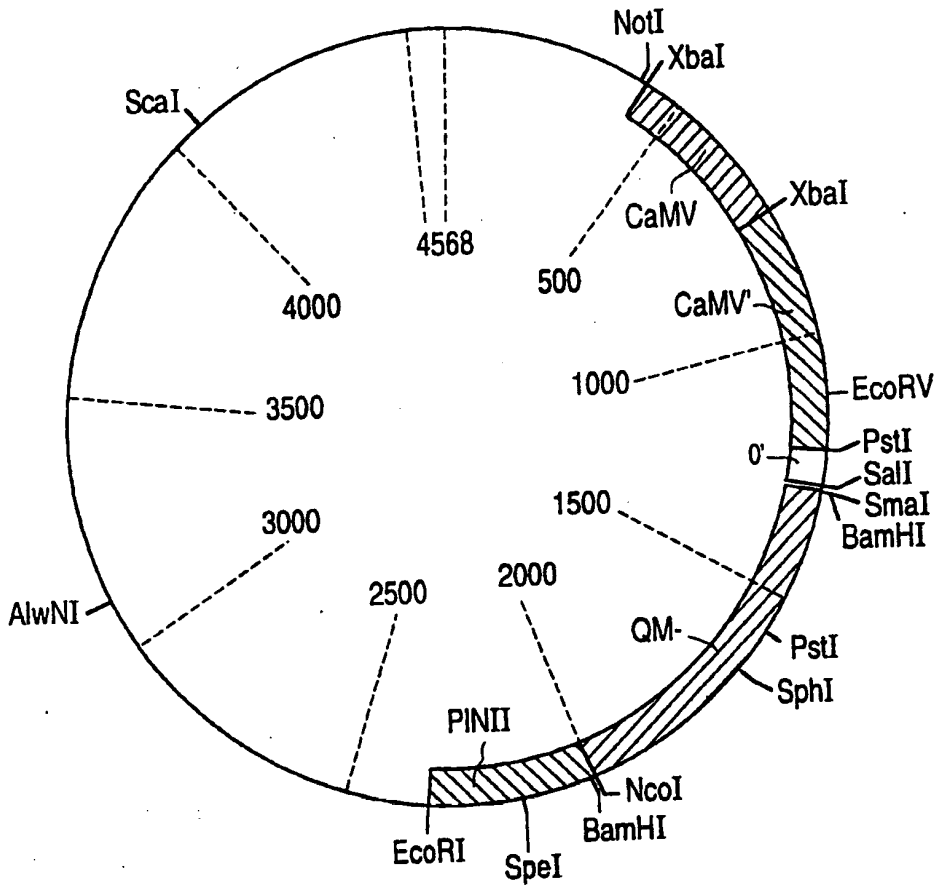
pPHI 3622 (4.717 KB)

FIG. 6



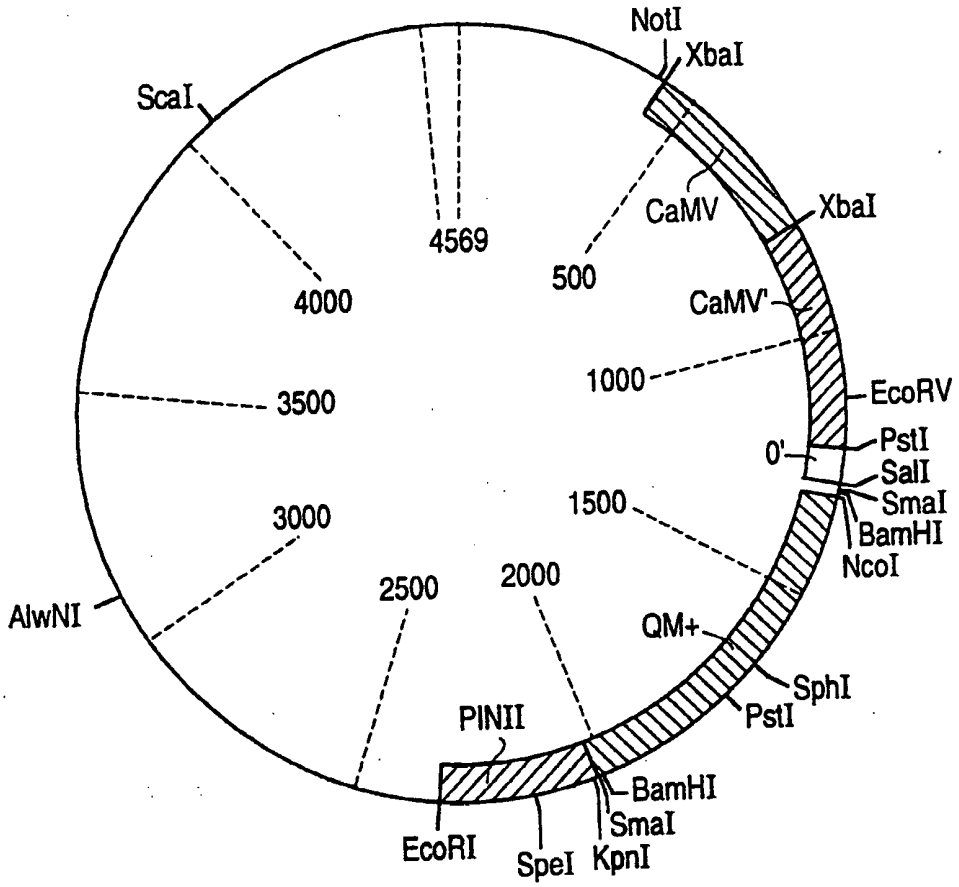
pPHI1285

FIG. 7



pPHI 4722 (4.568 KB)

FIG. 8



pPHI 4723 (4.569 KB)

FIG. 9

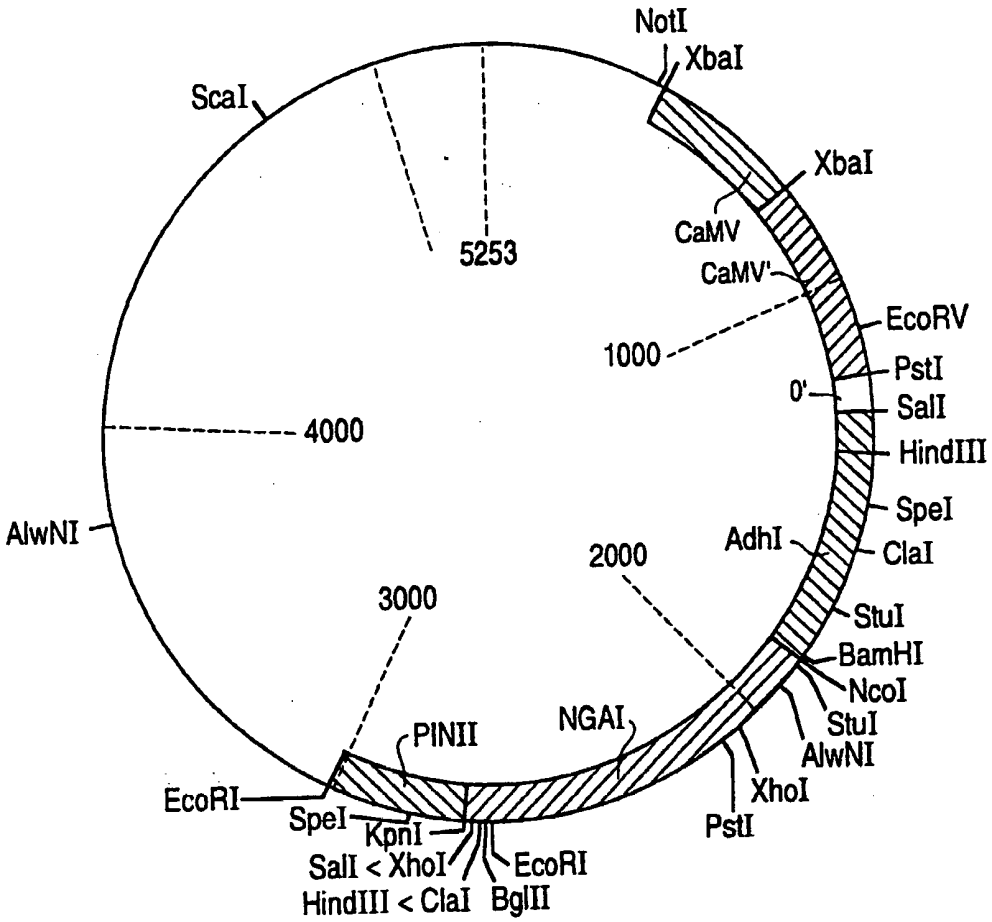
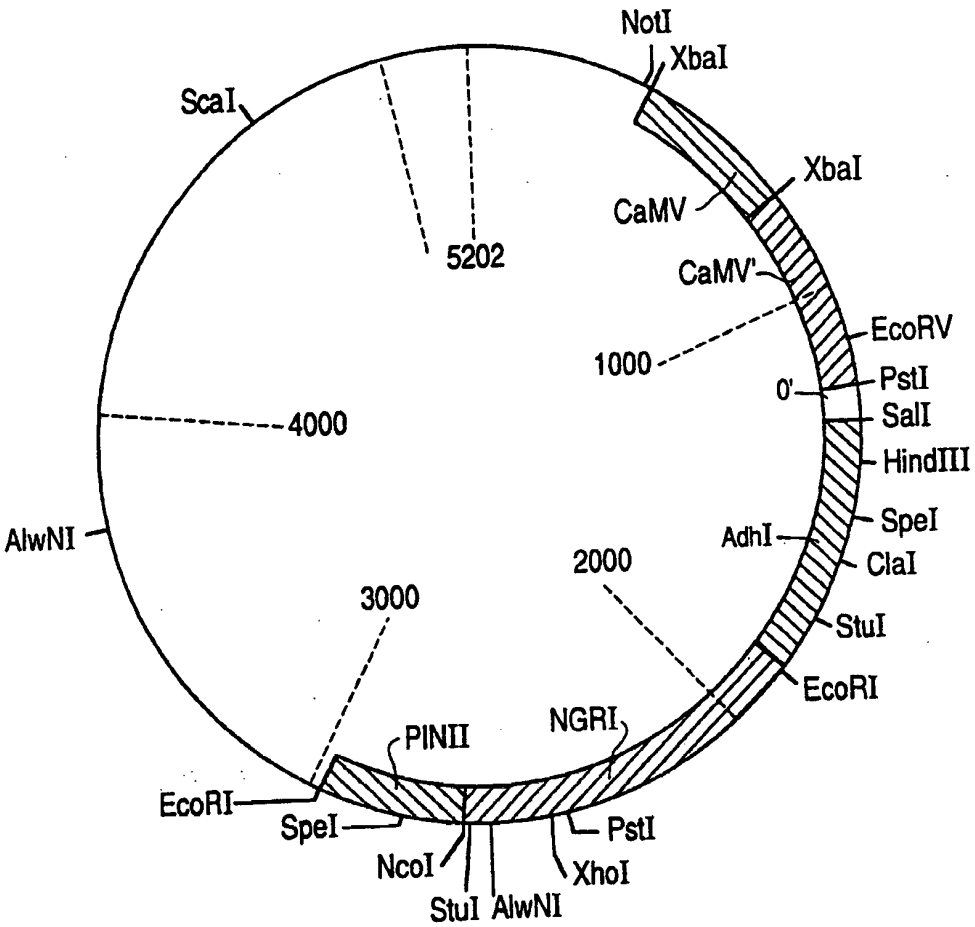

NotI
XbaI
ScaI
XbaI
5253
CaMV
CaMV'
EcoRV
1000
PstI
0'
SalI
4000
HindIII
SpeI
AlwNI
AdhI
ClaI
2000
3000
StuI
BamHI
NcoI
PINII
NGAI
StuI
AlwNI
XhoI
EcoRI
SpeI
KpnI
PstI
SalI < XhoI
EcoRI
HindIII < ClaI
BglII
pPHI 4719 (5.253 KB)

FIG. 10



pPHI 4720 (5.202 KB)

FIG. 11

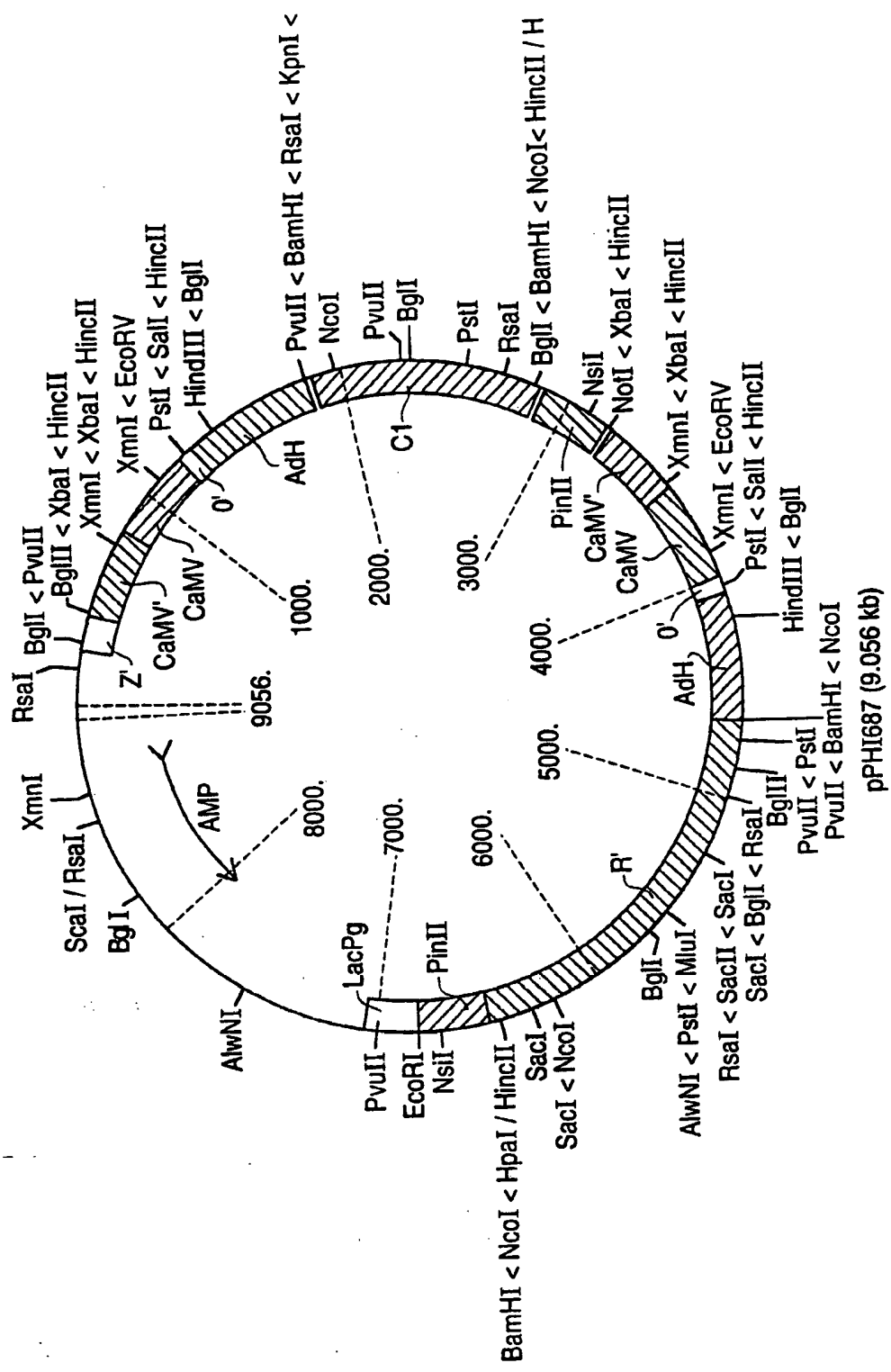
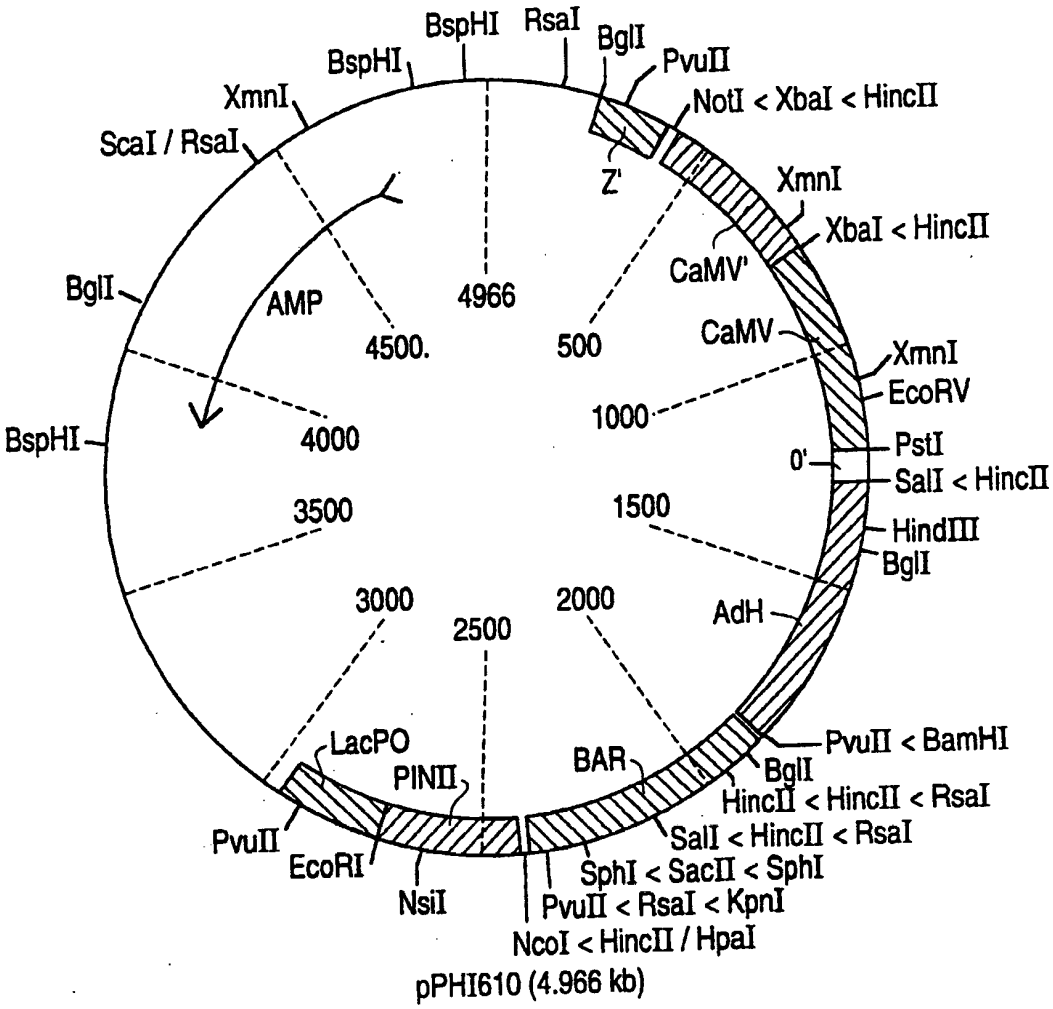
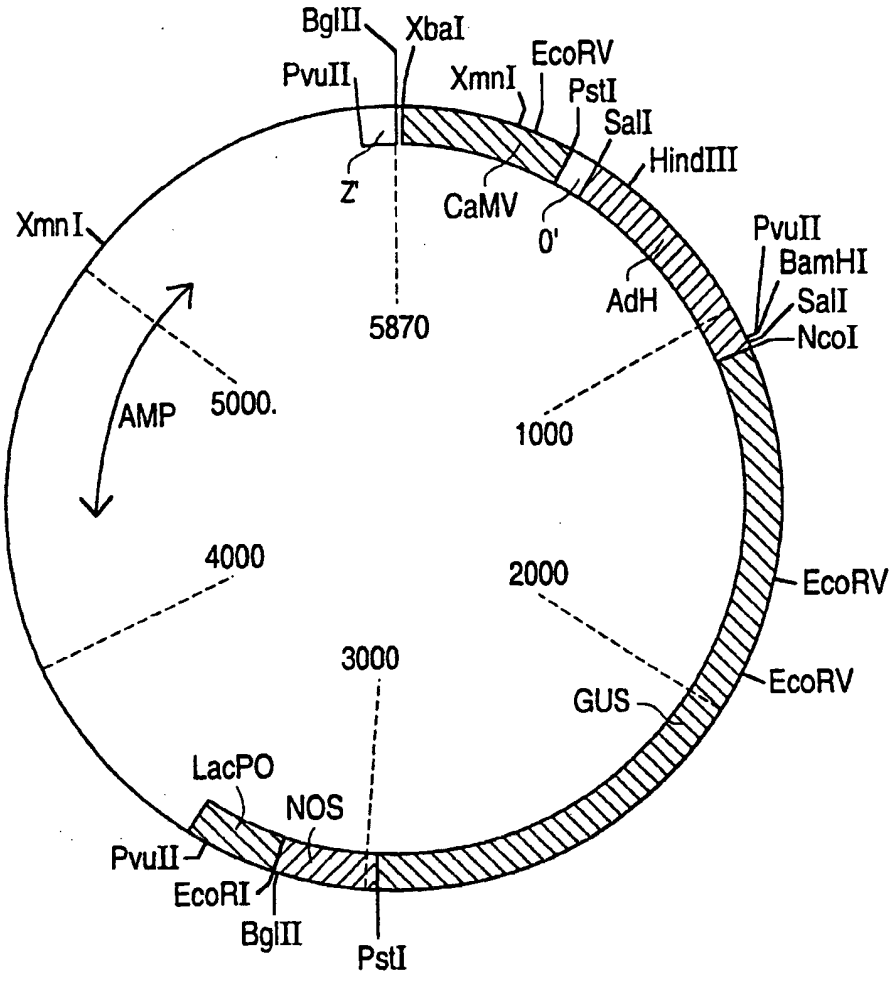

pPHI687 (9.056 kb)
0'
1000.
2000.
3000.
4000.
5000.
6000.
7000.
8000.
9056.
AdH
C1
PinII
CaMV'
CaMV
Z'
AMP
R'
LacPg
PvuII < BamHI < RsaI < KpnI <
NcoI
PvuII
BglI
PstI
RsaI
BglI < BamHI < NcoI< HincII / H
NsiI
NotI < XbaI < HincII
XmnI < EcoRV
PstI < SalI < HincII
HindIII < BglI
BamHI < NcoI
PstII
PvuII < PstII
BglII
RsaI'
SacI < BglI < RsaI'
RsaI < SacII < SacI'
AlwNI < PstI < MluI'
BglI
SacI < NcoI
SacI
BamHI < NcoI < HpaI / HincII
NsiI
EcoRI
PvuII
AlwNI
BglI
Bg I
ScaI / RsaI
XmnI
RsaI
BglI < PvuII
BglII < XbaI < HincII
XmnI < XbaI < HincII
XmnI < EcoRV
PstI < SalI < HincII
HindIII < BglI

FIG. 12



pPHI610 (4.966 kb)

FIG. 13



pPHI460 (5.870 kb)

FIG. 14

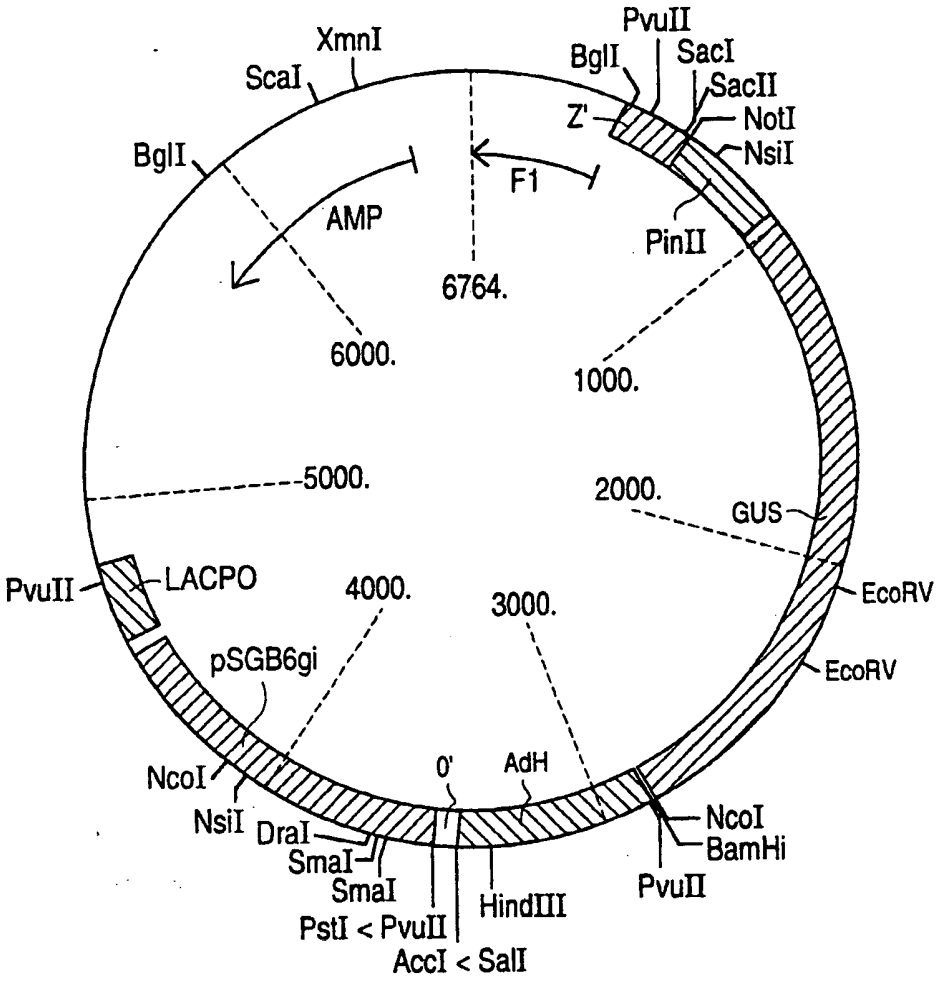
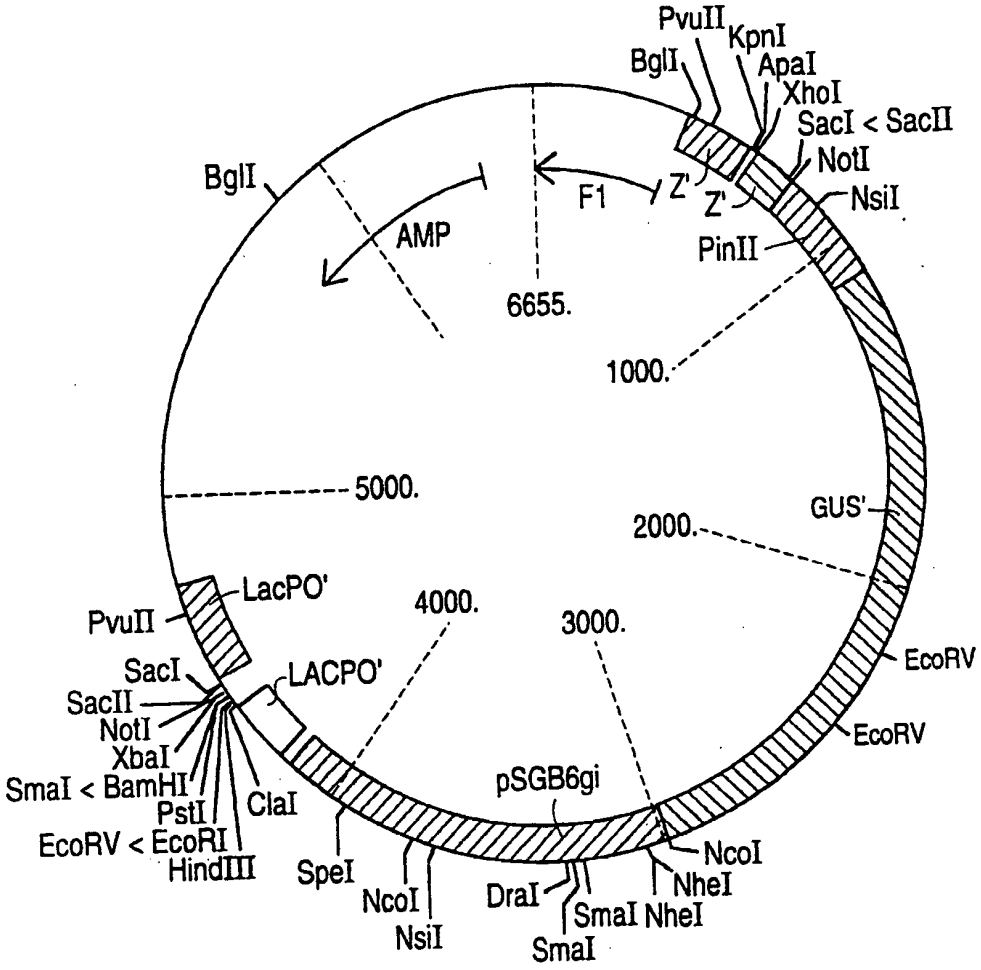
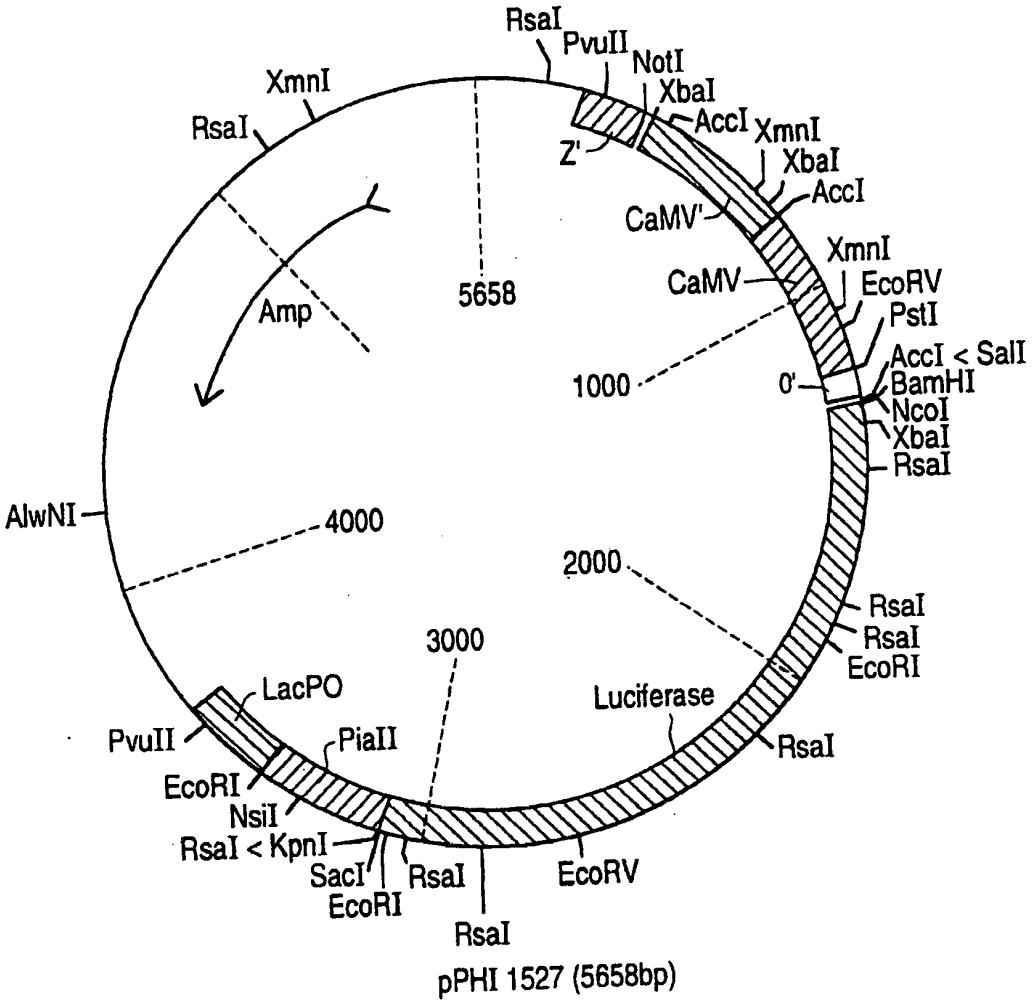

XmnI
ScaI
PvuII
BglI
SacI
SacII
Z'
NotI
NsiI
BglI
F1
AMP
PinII
6764.
6000.
1000.
5000.
2000.
GUS
PvuII
LACPO
4000.
3000.
EcoRV
pSGB6gi
EcoRV
NcoI
0'
AdH
NsiI
DraI
NcoI
BamHi
SmaI
SmaI
PvuII
HindIII
PstI < PvuII
AccI < SalI

FIG. 15



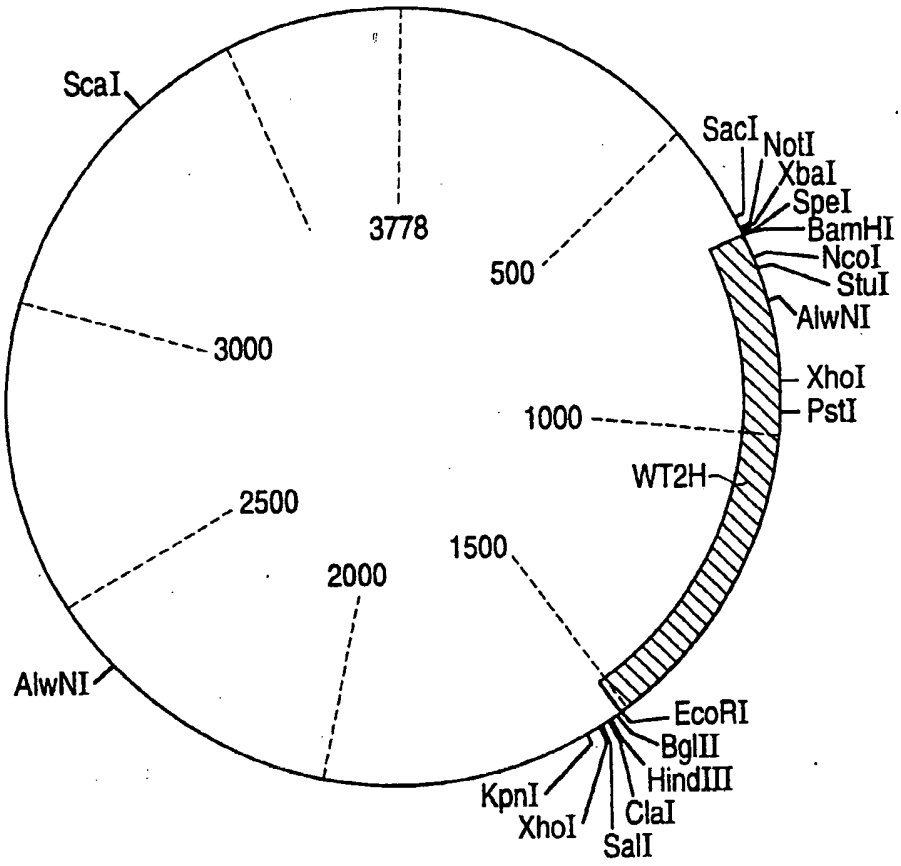
pPHI 2125 (6.655 kb)

FIG. 16



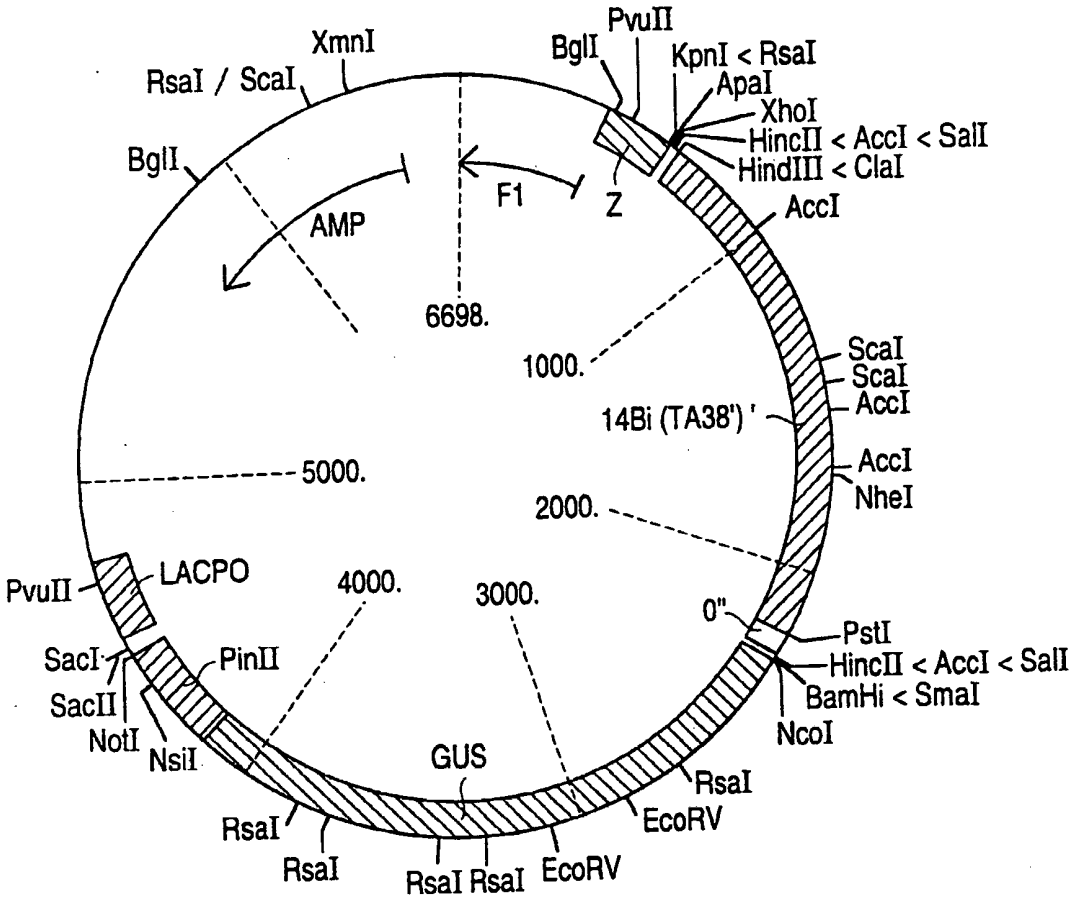
pPHI 1527 (5658bp)

FIG. 17



pPHI 3620 (3.778 KB)

FIG. 18



pPHI 1493 (6.698 kb)

FIG. 19

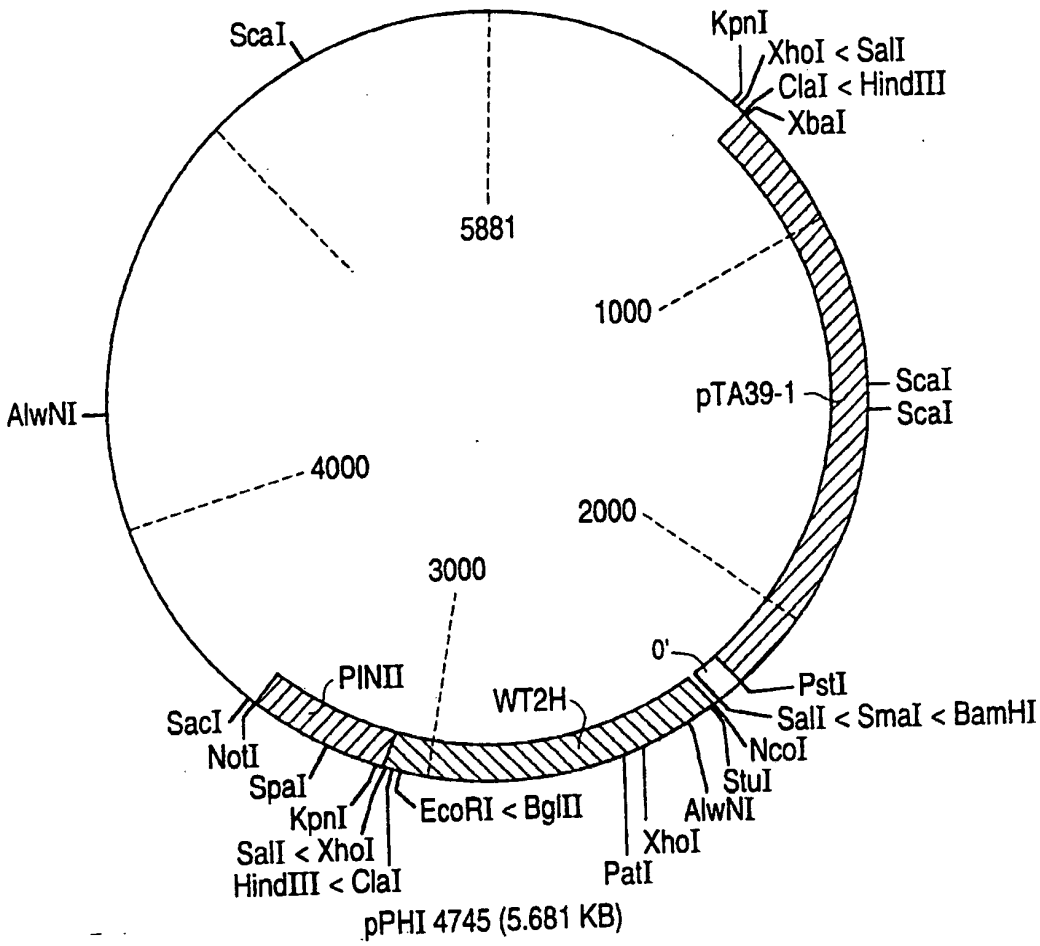
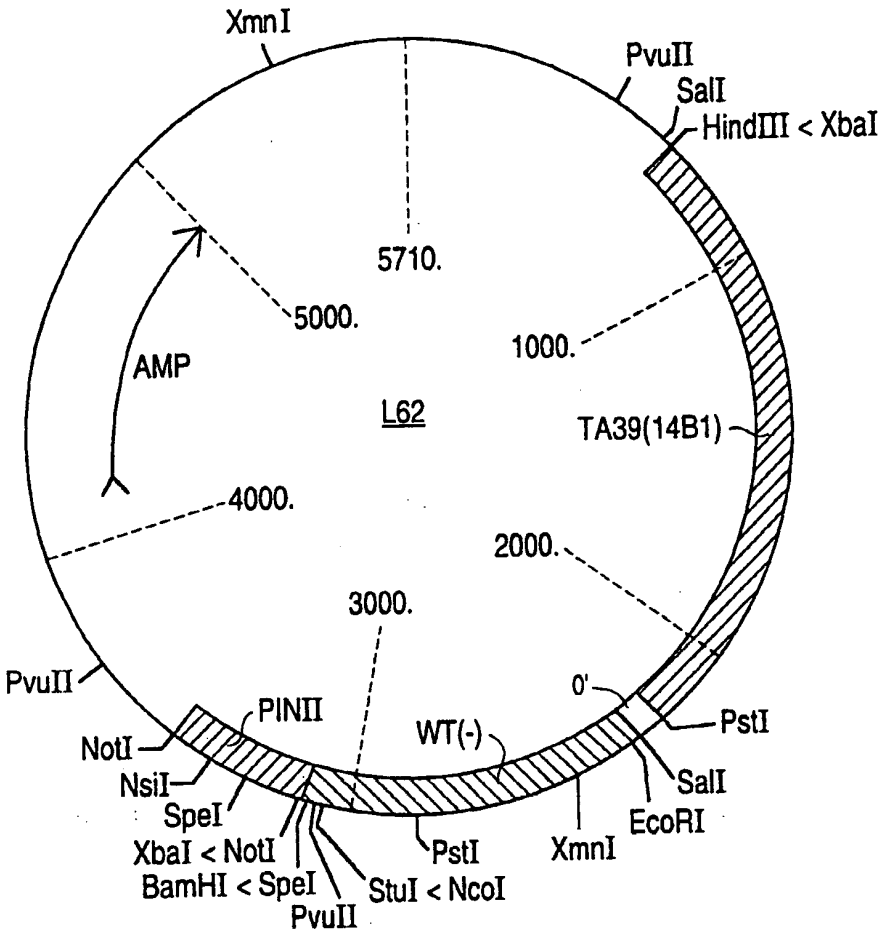
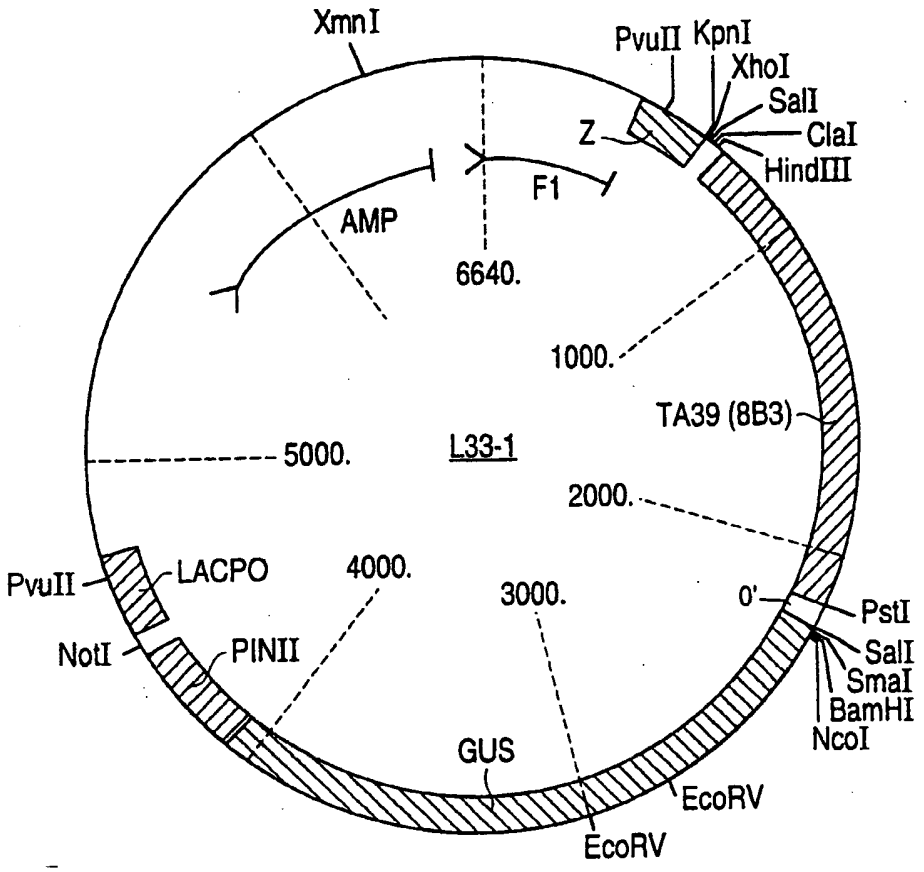

ScaI
KpnI
XhoI < SalI
ClaI < HindIII
XbaI
5881
1000
pTA39-1
ScaI
ScaI
AlwNI
4000
2000
3000
0'
PstI
SalI < SmaI < BamHI
NcoI
StuI
AlwNI
XhoI
PatI
WT2H
PINII
SacI
NotI
SpaI
KpnI
SalI < XhoI
HindIII < ClaI
EcoRI < BglII
pPHI 4745 (5.681 KB)

FIG. 20



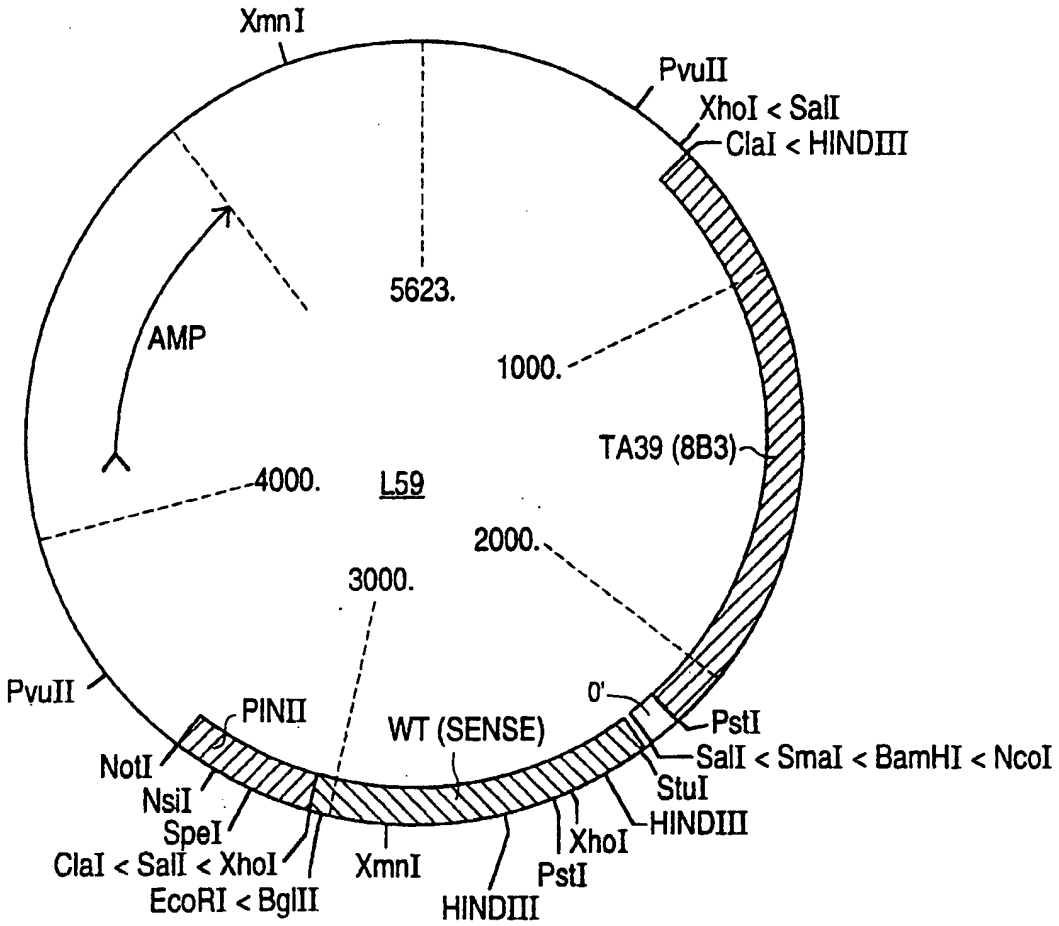
TA3914B1WT (-)

FIG. 21



pPHI 4855 (6.64kb)

FIG. 22



TA39 (8B3) : : maize wt

FIG. 23

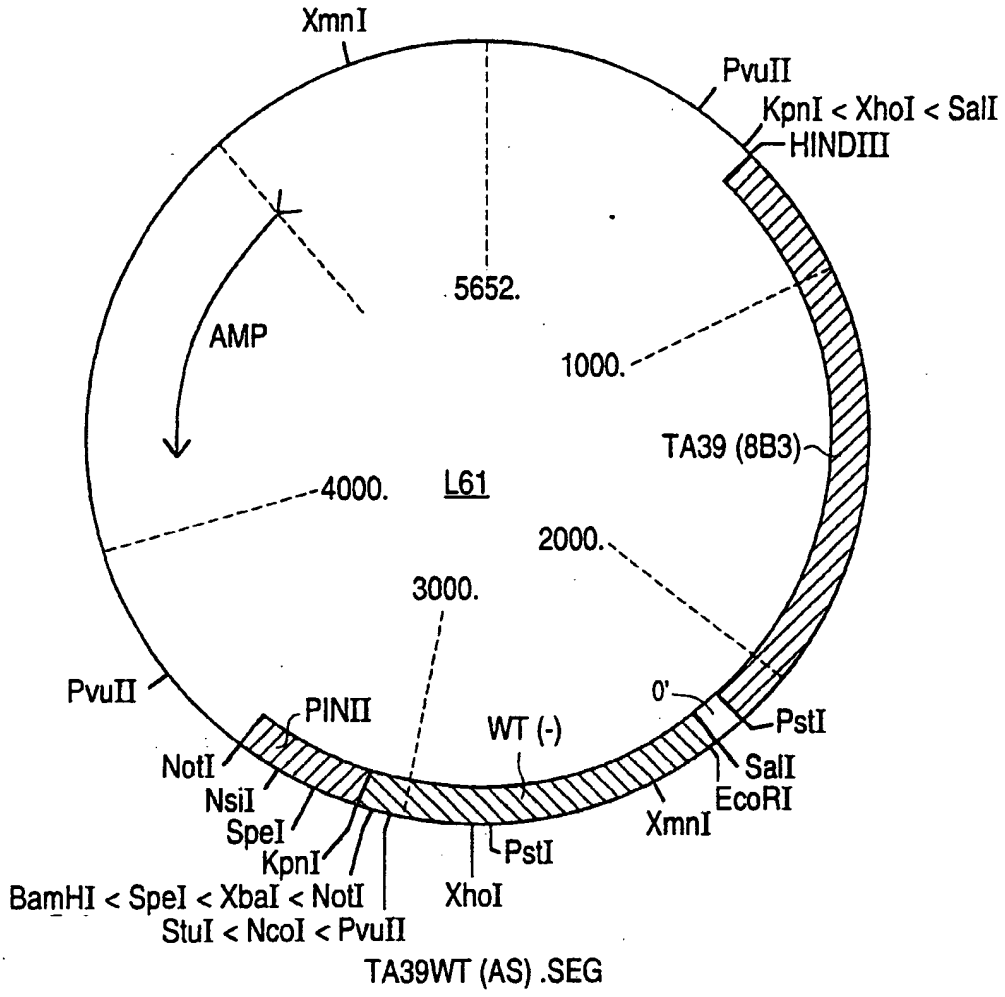

XmnI
PvuII
KpnI < XhoI < SalI
HINDIII
5652.
AMP
1000.
TA39 (8B3)
4000.
L61
2000.
3000.
PvuII
PINII
0'
PstI
WT (-)
NotI
SalI
NsiI
EcoRI
SpeI
XmnI
KpnI
PstI
BamHI < SpeI < XbaI < NotI
XhoI
StuI < NcoI < PvuII
TA39WT (AS) .SEG

FIG. 24A

```
   1 GTCGACCTGC AGGTCAACGG ATCGTTACAT AAACTGACAC ATTTCATCGT
  51 TATGTAACGA CAGATTTAAC GATACGATAC AATAAAATTT AAGTAACAAT
 101 CAAAACAAAC ATTGTATTTA AAGTAACAAT ACGATATGAT ACAATGGGTA
 151 ACAACCATCC AAACAAGCTG TAACAGAAAA CCCAAAAGTT CCTTCCATTT
 201 TCTCTTTAAT CCATCAAATT AGTTCACATT CCCCTTTATA TAAACTCAAG
 251 AACAACAACG CAACTTCAAA TTCATATTCT ACGATGGCCA TTCTTGCAAA
 301 AACTTCACCA ATTCATTCTC TATCTTTGGT TTTTCTCCTT GTTGCATTCA
 351 CCATAATCCA CCATGCCCAA AGCCAAAATA TTGGGCTATG TGTCATCAAT
 401 TGTGGCCAAC AAGCAATCAC TTGTGTCATC CGTTGCGGTC CGCCACCTCC
 451 ATCGGTTACT TGCTATCAAG GTTGCGCGAC TAGTGGTATC GGTTGTCTGA
 501 CCTCCTGTCT CACACCAGCT ACGCCACCAC CAACCACCCC ATCAGCGCCA
 551 ATGATCTTGG ACATGGGGAA AGATATCATT TAGGGAGTGA ATGCGAGCTT
 601 CAAATACTTG TTGTCTTGAC TCAAAATATA TATAATTGAA AACTCCTTAT
 651 ATTTTTATAT ATTAGCACCA TCTTCACATG GTGTAGTAAT ATTAGTTATT
 701 TTCAAGTTAA TATTCTGAAT CCGCCTCtCG TTATGAATAA ATAAAGCTTG
 751 ATGTAGATAT TTGAGAGGCT TTTGTTATAA TAGTGTTAGC TTGCATGTTT
 801 GAACTTTGAA CTCTTGTATG GGAGGGTTGT AATGATTGGG CTGATATGAG
 851 ATGGTACTGG GCGAACTGCA CCAATAGCCA CTTTTAAGTT TGCTATAAAA
 901 AATGTATTCA TAATTTATAA TGTATTTAAA GATTAGCCAA TTCGTCTAAA
 951 CTTAAGGACT AAATATATAT CCTGAATTTT TGAGCTGCTA ATTTGAAATT
1001 CAGGACTAAG TGTCCTAAAT TTTTGAACTG CTAATTTAAG ATTCAGGACA
1051 TAAGATTCGA ATTTCTGGAC ACGATTTTCG AACTTTAGGA CACGATGTCC
1101 TGAAGTTTGA ACTTTGAGGT TTAAACTTTA GGACGTCATG CCCTAAAATT
1151 TGAGTAAATT AGTTAATCTT TAAATATATT ATAAAGTATA AATACATTTT
1201 AAATAACAGG CTTAAAAGTG ACTACCCGCT ATAATTTCTA CGAAGGCCCT
1251 GCGGATATTA GTATCATGTA GCTTTTGTTG AACTCTTCGC ATACATTCAA
1301 GTGTAATAAC CAAGTTTATT CTTTGGGTAT ATAAAGTGGT CCACTATTTA
1351 ATATTCTTAC AATATAATTG TAAATTGAAT TTCAATTTTT CTTTTTTATT
```

FIG. 24B

```
1401 TAGTCAAAGT ACTTTATACT TATGTACTTG ACGTCATAGC GAGTTTTGTG
1451 CTATTTTTCC CTTTAGCAAA GTACTTCAAT TTCACATATT TGAACCTAAG
1501 ACTCAATTTT CTCTAGTTAA AAAGAACCGA ACTAAAAAAA ATTCTCATTT
1551 TATCTACGAA TAATTTTACT TTTCCCTTTA AGAGAACCAA CAAAACATGC
1601 AAAATAACTT TTAGGTAACG TTTCTAAACT TACTAAATCA TATAAATCTC
1651 TAACTTGTAG GATTTTAGCA TAACAATATG TTAATAATAT GTTTTGTGAA
1701 AAATTCTGTA TACGCCATTG TTCGGGCCAG AGTTTTGAAT CATAACGCGG
1751 GCCAAGAAAA CGTGCACCAA TTCCAGGCCG ACAAAAAATA AAGTTTCTTG
1801 AAAGAAAAAA TAATAATGTA TAGTTGCTCT CAAAATAATA GCCGAAAAAA
1851 TATATTTTTG TATGTATACA AGAAATATAC AAATTTCATA CACTTTTTCG
1901 GCTAGTGAAT ATAAAAACTT TCAGCCGCGA GCTAAATATG ATCTTTGCCG
1951 TTGTTAAAAT TGGTCAAATT ACTATAAACA AAGTCCAAAA GATAATAATA
2001 ATTCAACCCC TTTTATCCGT AATGGTGAGT AACCCTCACG AATTATATTC
2051 AAATGGGATT GCCGTTCAAC AAGAATAGTT CACATCTTTT GTATGAACAA
2101 AGTTGTCCAT AACAGCAAAA ACTCTTTTCT ACACCCCCCC CCCCCCaccC
2151 CCACCTCCCT ATATAATGTC ACGTCAAGTT TATGTTTTCG TATCATTTTC
2201 TTGAAAAGAT ATTATATCAT CATAGAAGCA TCAAGTGATA AGGCAACGGA
2251 TTTTGGACCC ACTAATTCCA ATATGGCTTT TCTTGCAACA ACTTCACCAA
2301 TTCATGTTTT AGCTTTGCTT TTGCTTATTT TTGCATCTAC CAAAATCCAC
2351 CATGCTCAAG GTAAATCAAT TACAGGGCCA TGTGTGGTTG CTTGTAGCAA
2401 AAAAACAATC GCTTGTGTTG TTAGATGCAG ATTTGCGACA GATAAATGCT
2451 CTCAGGATTG TGCGATTGAT AGTATCCATT GTGTGAGTTC GTGTCTCCTT
2501 CAGAACTCGT CATCACCACC GGCTATGATT TTGGACACGG ACGACAGTCG
2551 GGACAGAGCT AGCATGAAAC ATATCAGTAA CTTCGATCTG AACCCTATGT
2601 ATTAGTTAAA AAAATCCACT GAATGTGGAC AAATAATTAC AATAAACTCC
2651 ATAATCCATA AGCTTCAATA TATAAGTTAT TAACTTTAAA TCCTGGATCC
2701 ACCTCTACGA ATCTCGATAT ACTTATTATT TAGGGAGTGG GTGTGTGCTA
2751 GACACTCACG GTCTTTGATT CAAGATGATA AAGTTGAAAA CTTTTATATT
2801 TGTACTAAGT TAGCACCATC ATCAAAACTA AATAATGGTG TAGTAGTATT
2851 TTGCATTCAC GGCAAATAAA ATAGTAGTAT TTTGTATTTA TTGATTTGTT
2901 GGTAAAATTA CAAGTAATTG CAGTTGAAAA ATAAGATGAA TAAAAATAAA
2951 TAAATattct ccaggctaaa gcgtgaatat ctcgctctct naaaggagat
3001 tcaagcccac tgcggnaaca cctccaccgg nccagcaata atactctcga
```

FIG. 25A

```
   1 CCCACCATGC CCCAAGGCCC AAAATATTGG GCCTATGTGT CCATCAATTG
  51 TGCCCAACAA GCAATCACTT GTGTTATCCG TTGCGGCCCG CCACCTCCAT
 101 CGGTTACTTG CTATCAAGGT TGCGCGACTA GTGGTATCGG TTGTCTGACC
 151 TCCTGTCTCA CGCCAGCCAC ACCACCACCA ACCACCCCAC CAGCGCCAAT
 201 GATCTCGGAC ATGGGAAGAG AAATCTTTTA GGGGGTGAAT GCGAGTTTCA
 251 GATACTTGTT GTCTTGACTC AAAATATATA TAATTGAAAA CTTTTATATT
 301 TTTACTATGT TAGCACCATC TTCACAAAAT GGTGTAGTAA TATTAGGTAT
 351 TTTCAAATAA ATATTCTAGA TCCGTCTCTG TTATAAATAA ATAAAGCTTG
 401 ATCTAGATAT TTGAGAGGCT TTGTTATAAT AAAGTTAGCT TGCTTGTTTG
 451 AACTTCTAAC TCTTGTATGG GAGGTTTGTA GTCGTATCAC TAATATCAGA
 501 AGATTATAGA AACATTGGGA ATAAAATCAT TCGGGCCCGA AGATAATGAT
 551 TGGGATGATA TGAGATGGCC CTGGGCGAAG TGCACCAATA GCAACCTTGA
 601 AGTCTGATAT AAAAAATATA TTAATAATTT ACGATATATT TAAAGATGGG
 651 TCAATTCATC TAAGTTTCAG GAGTAAGTAT ATATCCTGAA TTTTTGTATA
 701 CTGAACTTTT GAATAGCTAA TTTGAAATTC AGGACTAAGT GTCCTAAATT
 751 TCTGAACTGC TAATTTAATG TTTAGGATAT AAGATTCGAA TTTTTGGACA
 801 CGATTTTTGA ACTTTAGGAC ACGATGTCAC GAAATTTGAA TTTTGAGGTT
 851 TAAACTTCAG GACGTCGTGT CCTGAAATTT AAACAAATTA GTTAATCTTT
 901 AAATATATTA TAAATTATAA ATATATTTTA AATAACATGC TTATAACGGA
 951 TGTTAGTATC ACGTAGCTTT TGTTGAACTC TTCGCATACA TTCAAGTGTA
1001 ATAACCAAGT TTATTCTTTG GGTATATAAA GTGTTCCACT ATTTAATATT
1051 CTTACAATAT AATTGTAAAG TGAATTTCAA ATTTTCTTTT TTATTTAGTC
1101 AAAGTACTTT ATACTTATGT ACTTGACGTC TTAGTGATTT TTGTGCTATT
1151 TTTCCCTTTA GCATAAGTAC TTCAATTTCA CATATTTGAA CCTAAGACTC
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FIG. 25B

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1201 AATTTTCTTT AGTAAAAAAA GAACCGAACT AAAAAAATTC TCATTTTGTC
1251 TACGAATAAT TTAACTTTTC CCTTCAAGAG AATCAACAAA ACATGCAAAA
1301 TAACTTTTAG GTAACGTTTC TAAACTTACT GAATCATAGA AGTGTCAACT
1351 TGTAGGATTT TAGCATAACA ATATGCTAAT AATATtTTAT GTGAAAAATT
1401 CTGTATACGC TATTGTTCGG GCTAGCGTTT TGAATCATAA CGTGGGCAAA
1451 GAAAATGATG CACCAATTCC AGGCCGACAA AAATTAAAGT TTCTTGAAAG
1501 AAAAAATGAT ACTGTATAGC TGCTCTCAAC ATAATAACCG AAAAAATATA
1551 TTTTTGTATG TTATATACAA GAAATATATA AATTTTATAT ACCTTTTCGG
1601 CTAACGAATA TAAATATTTT CAGCCGCGAG CTAAGTATGA TCTTTGCCGT
1651 TGTTAAAATT GGTCAAATTA CTATAAACAA AGTCCAAAAG ATAATAATAA
1701 TTCAACCCCC TTTATCCGTA ATGGTGAGTA ACCCTCACGA ATTATATTCA
1751 AATGGGATTG CCGTTTGTAA AATCtGCCTT CTTTACTCTA ACAAGATTAG
1801 TTCACATCTT TTGTATGAAT AAAGTTGTCC ATAACAGAAA ACTCAAAACT
1851 TTGCTCTACA TCCCTCCTAT ATAATGTCAC GTCAAGTTTA TGTTTTCGTA
1901 TCATTTTCTT GAAAAGATAT TATATCATCG TAGAAGAACC AAGTGGTAAG
1951 GCAACGGATT TTAGTCCGGC TAATTCCACT ATGGCTTTTC TTGCAACAAC
2001 TTCACCAATT CATGTTTTAG CTTTGCTTAT GCTGATTTTT GCATCTACCA
2051 AAATGCACCA TGCTCAAGGT AAATCAATTA CAGGGCCATG TGTGGTCGCT
2101 TGTAGCAAAA AAACAATCGC TTGTGTTGTT AGATGCAGAT TTGCGACAGA
2151 TAAATGCTCC CAGGATTGTG CGATTGATAG TATCCATTGC GTGAGTTCTT
2201 GTCTCATTCA GAACACGCCA CCACCAACGG CTATGATTTT GGACACGGAC
2251 GACAGTCGGG ACGGAGATAG CATGGAATAT ACCAATAACT TCGATCTGAA
2301 CCCTATGTAT TAGTTGAAAA ATCCACTGAA TGTGGACAAA TAATAAACTT
2351 TAGAACCCAT AAACTTGATT ATATAAGTCA TGAAATTCAA ATCCTGAATA
2401 CGCCTCTACG AATCTAAATA TACTTATTAT TTAGGGAGTG GGTGTGTGCT
2451 AGACACTCAC AGTCTTTGAT TCAAGATAAT AAAGTTGAAA ACTTTTATAT
2501 ATGTACTAAG CTAGCACCAT CATCAAAACT AAATAATGGT GTAGTAGTAT
2551 TTTGCATTCA CGGAAAATAA AATAGATGTA TTTTGTATTT ATCAATTTGT
2601 TGGTGAAATT ATAAGTAATT ACAGTTAAAA AATAAGATGA AGAAAAATAA
2651 ATAAATATTC TTCATGCTGA AGCGCGGATA TCTCGCTCTC TTTAAGGAGA
2701 TTCAAGCCCA CTGCAGTAGT ATCTTCACCG GTCCAGCAGT AATACTCTTG
2751 ACTTGTCCCC TCTAGGATAC AACAGCTCGA CAATATCGTT TGACTCGAAC
2801 AAATTCTGGA CAAGTTGTAG TTTCCAAAAC TCCACCAAAA TGAACACCTT
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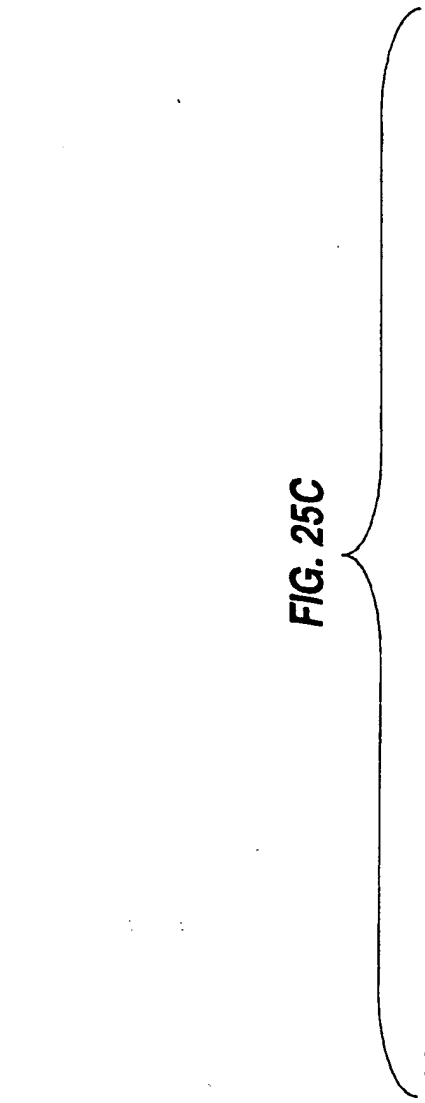
2851 CCCGCAACAG AAATAATATT CTCTTTTGTC GAGAATAAGT TGGAGACTTA
2901 GATTTTCTCC CTCTATTAAC TCTCCTTTTC ACAACACCAA GTTCATTTTT
2951 TTCATACACA CTACCAAATT TTCCCATTAC AAACACAAGG CAGTGTCACG
3001 ACCCGAAATT CCCAATCTCG GGATCGTGAT GGCGCCtaac attccacttg
3051 ctaggcaagc caacattaga gtaaatctta atatatttta aaataattca
3101 aataattaaa atcaattaaa ctgaaataaa actggaataa agtgcgaaaa
3151 gttataaaaa ccaaaatatc taagtacaac cccggatcc

FIG. 25C

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/82 C12N15/29 C07K13/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL SEQUENCE DATABASE REL.33 ACC. NO. Z15157 (12-10-92) A.thaliana mRNA for Wilms' tumor suppressor homolog ---	1-5,7,8
X	EMBL SEQUENCE DATABASE REL.31 ACC. NO. X64621 (28-2-92) O. sativa R22 mRNA ---	1
P,X	PLANT PHYSIOLOGY vol. 102 , May 1993 pages 329 - 330 RIVERA-MADRID, R., ET AL. 'Nucleotide sequence of an Arabidopsis thaliana cDNA clone encoding a homolog to a suppressor of wilms' tumor' see the whole document --- -/--	1-5,7,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1994	- 4. 08. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO,A,92 09685 (AUSTRALIAN NATIONAL UNIVERSITY) 11 June 1992	

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		EP-A-	0559729	15-09-93
		JP-T-	6504430	26-05-94